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Document Processing Center (TS-790)
Office of Toxic Substances
U.S. Environmental Protection Agency
401 M Street, S.W.
Washington, D.C. 20460
ATTN: 8(e) Coordinator

Dear Sir:

SUBJECT: ACUTE TOXICITY OF EPIKOTE® 862 TO ONCORHYNCHUS MYKISS, DAPHNIA MAGNA, AND SELENASTRUM CAPRICORNUTUM.

The following information is submitted under TSCA 8(e).

The toxicity of EPIKOTE Resin 862 (CAS Number 28064-14-4) to rainbow trout (Oncorhynchus mykiss), freshwater zooplankter (Daphnia magna), and freshwater unicellular alga (Selenastrum capricornutum) was determined by exposure of the organisms to test media prepared from saturated solutions of the test substance. Note that the concentrations of the test substance varied with time. The reported results are:

1. Oncorhynchus mykiss: The LC_{50}, 96-hours, was calculated to be 0.55 mg/liter,

2. Daphnia magna: The EC_{50}, 48 hours, was found to be 1.6 mg/liter,

3. Selenastrum capricornutum: The EC_{50}, 72 hours, was 1.8 mg/liter.

Attached is a copy of the report SBGR.92.237 entitled, "EPIKOTE 862: Acute Toxicity to Oncorhynchus mykiss, Daphnia magna and Selenastrum capricornutum.

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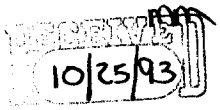

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This report has been compiled based on information available as of the date of filing. The corporation, its agents and employees reserve the right to supplement the data contained in this report, and to revise and amend any conclusions drawn therefrom.

This report contains no confidential business information.

Very truly yours,

R. N. Shulman
General Manager
Health, Safety, and Environment
Shell Oil Company

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GROUP RESEARCH REPORT

SBGR.92.237

500 70 292 RE 9201

EPIKOTE 862: Acute toxicity to Oncorhynchus mykiss, Daphnia magna and Selenastrum capricornutum

L.E. Wyness

SICC, CMKP

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EPIKOTE* 862: Acute toxicity to Oncorhynchus mykiss, Daphnia magna and Selenastrum capricornutum

(Experiment Number 4607)

SUMMARY:

The acute toxicity of saturated solutions of EPIKOTE 862 and dilutions of it has been determined to the fish Oncorhynchus mykiss (rainbow trout), the crustacean zooplankter, Daphnia magna and the unicellular alga, Selenastrum capricornutum.

Saturated solutions of EPIKOTE 862 were prepared by mixing EPIKOTE 862 with dilution water/culture medium for 24 h, after which the contents were allowed to settle and the aqueous phase drawn off and either used or further diluted before use in the toxicity test. For all of the toxicity tests, saturated solutions of EPIKOTE 862 were prepared at 1000 mg/l.

For the test with O. mykiss, dilutions of the saturated solution of EPIKOTE 862 resulted in a series of mean measured concentrations from 4.4 to 0.1 mg/l. By the end of each exposure period the concentrations fell by an average of 11 to 25% of the initial measured concentrations.

For the test with D. magna, dilutions of the saturated solution of EPIKOTE 862 resulted in a series of initial measured concentrations from 7.4 to 0.24 mg/l. By the end of the 48 h exposure period the concentrations fell by 8 to 26% of the initial measured concentrations.

For the test with S. capricornutum, dilutions of the saturated solution of EPIKOTE 762 resulted in a series of measured concentrations from 10 to 0.31 mg/l. By the end of the 72 h exposure period the concentrations fell by 34 to 44% of the initial measured concentrations.

The acute toxicity of EPIKOTE 862 to O. mykiss was determined in a 96 h semi-static, sealed toxicity test with daily renewal of the test media. Based upon mean measured concentrations of EPIKOTE 862 in the test media over the relevant exposure periods the 24, 48, 72 and 96 h LC_{50} values have been calculated to be respectively 1.1, 0.73, 0.55 and 0.55 mg/l.

The acute toxicity of EPIKOTE 862 to D. magna was determined in a 48 h sealed static toxicity test without renewal of the test media. Based upon mean measured concentrations of EPIKOTE 862 in the test media over the duration of the test the 24 and 48 h EC_{50} values have been calculated to be respectively 3.2 and 1.6 mg/l.

* Shell trade name

The toxicity of EPIKOTE 862 to S. capricornutum was determined in a 72 h sealed growth inhibition test in which the test medium was not renewed. Based upon mean measured concentrations of EPIKOTE 862 in the test media over the duration of the test and an analysis of the concentration of chlorophyll a in the test media at the end of the test the 72 h EC_{50} has been calculated to be 1.8 mg/l. The NOEC of EPIKOTE 862 to S. capricornutum was calculated to be 0.24 mg/l.

C. L. Volger

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Date: 5/7/93

[redacted]

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TEXT:

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1. INTRODUCTION

The acute toxicity of EPIKOTE 862 to the fish, Oncorhynchus mykiss, (rainbow trout) the crustacean zooplankter, Daphnia magna, and the planktonic alga Selenastrum capricornutum was determined. These data were collected within the framework of the assessment of hazard associated with the manufacture, transport, use and disposal of EPIKOTE 862.

2. MATERIALS AND METHODS

2.1 Sample

The sample of EPIKOTE 862 was received from Shell Nederland Chemie B.V. and was given the laboratory reference number ST92/100 (Appendix A).

2.2 Toxicity tests

2.2.1 Test organisms

Oncorhynchus mykiss Walbaum

Healthy O. mykiss fingerlings were obtained from Zeals Trout Farm, Wiltshire, (Batch No. RT/117) and acclimated to the test conditions for more than nine days before use (Appendix B; Appendix C). A sample of ten of the fish used in the test had a mean length of 2.9 cm (range 2.8 to 3.2 cm) and a mean weight of 0.24 g (range 0.16 to 0.34 g). The size of the fish were below the size range recommended in the OECD guideline 203 5 ± 1 cm (fish acute toxicity test). However, the difference between the recommended and actual size would not have affected the outcome of the test.

Daphnia magna (Straus)

D. magna, less than 24 h old, were taken from a laboratory culture. The culture is derived from a strain obtained (via ICI Brixham Laboratory) from I.R.Ch.A., France (Appendix B; Appendix C).

Selenastrum capricornutum (Prinz)

S. capricornutum were taken from a semi-axenic laboratory culture. The culture is derived from a strain (ATCC 22662) obtained from the American Type Culture Collection, Maryland, Ohio, U.S.A. (Appendix B; Appendix C).

2.2.2 Preparation of test media

EPIKOTE 862 was not wholly soluble in water and did not form a homogeneous and stable dispersion on contact with water. Saturated solutions of EPIKOTE 862 were prepared by mixing 1000 mg/l EPIKOTE 862 with dilution water/culture medium appropriate to each test (Appendix C) for 24 h in sealed vessels with minimum headspace. As a result of the components of EPIKOTE 862 having similar solubilities in water, dilutions of the saturated solution were prepared at 500, 250, 125, 31 mg/l for the D. magna and S. capricornutum tests, and in addition, 15.6 mg/l for the test with O. mykiss, in dilution water/culture medium. After mixing the contents of each vessel were allowed to stand for 1 hour before drawing-off the aqueous phase for subsequent testing.

2.2.3 Test methods

The range of concentrations used in the definitive tests that are described below were based upon the results of range-finding work.

O. mykiss: A sealed 96 h semi-static toxicity test was carried out with daily renewal of the test media. The test vessels were 11 litre full-volume, spherical, glass reaction vessels which were completely filled with test medium. Quantities of dilutions of the saturated solution of EPIKOTE 862 were added to the test vessels to give an approximately geometrically spaced series ranging from 500 to 15.6 mg/l. An additional vessel received no EPIKOTE 862 and served as a control. Ten O. mykiss were placed in each vessel. The vessels were then sealed with glass plates ensuring that all air was excluded. The fish were not fed during the test.

At points throughout the test (3, 24, 48, 72 and 96 h) the fish were observed and the numbers exhibiting toxic symptoms were recorded. The symptoms were classified into one of five categories from (a) - no toxic symptoms to (e) - dead (See Table 1). At 24 h intervals any dead fish were removed and surviving fish were transferred to freshly prepared media. The dissolved oxygen concentration and pH of the media in each test vessel were determined at the start and end of the test and prior and subsequent to each renewal of the test media. The total hardness and residual chlorine concentration were determined in all fresh test media. The temperature of water in an aquarium adjacent to the test vessels was determined at hourly intervals throughout the test.

D. magna: A sealed 48 h static toxicity test was carried out without renewal of the test media. The test vessels were 150 ml Erlenmeyer flasks which were completely filled with test medium. Quantities of the saturated solution of EPIKOTE 852 and dilutions of it were added to the flasks to give approximately geometrically spaced series of application rates ranging from 1000 to 31 mg/l. There were two replicate flasks at each test concentration. Two further flasks received no EPIKOTE 862 and served as controls. Ten D. magna, less than 24 h old were added to each flask. The flasks were then sealed with black screw-on lids that excluded air from the vessel. After 24 and 48 h the numbers of immobilised D. magna were recorded. D. magna were considered to be immobile if, when the contents of the flask were briefly swirled they did not swim during a 15 second period of observation. The pH and concentration of dissolved oxygen were determined in each of the test media at the start and end of the test. The total hardness of the control medium was determined at the start of the test. The temperature of water in a flask adjacent to the test vessels was determined at hourly intervals throughout the test.

S. capricornutum: A 3 day sealed growth inhibition test was carried out. The test vessels were 300 ml full-volume Erlenmeyer flasks which were completely filled with test medium. Quantities of the saturated solution of EPIKOTE 862 and dilutions thereof in algal growth medium were added to the flasks to give approximately geometrically spaced series of application rates ranging from 1000 to 31 mg/l. There were four flasks at each concentration. Seven further flasks received no EPIKOTE 862 and served as controls. Three out of each set of four flasks containing EPIKOTE 862 and

six of the control flasks were then inoculated with sufficient S. capricornutum cells to achieve a concentration of 5000 cells/ml. The cell concentration in each flask was then checked using a Coulter Multisizer (Appendix B). The remaining flasks (one control and six flasks containing EPIKOTE 862) were not inoculated and were used to determine particle counts in the absence of S. capricornutum. Two glass marbles were placed in each flask to ensure good mixing during incubation. The flasks were sealed with glass stoppers and then incubated in a cooled orbital incubator (100 cycles/min) under constant illumination (~3000 lux) at a nominal temperature of 22-26°C.

At the end of the test the growth of the cultures in the flasks was determined by measuring the chlorophyll a concentration of a sample taken from each flask. Chlorophyll a concentration was determined by boiling the sample with methanol, filtering it and measuring the absorbance of the filtrate with light of wavelength 665 nm and 750 nm (Talling and Driver 1963).

At the start and finish of the test the pH of each test medium was determined using the method described in Appendix C. The temperature in the test incubator was monitored at hourly intervals throughout the test.

2.3. Chemical analysis

For each of the toxicity tests samples of the test and control media were analysed at the start and conclusion of each exposure period to determine the concentration of EPIKOTE 862 present. Full details of the sampling and analytical method procedures are presented in Appendix D.

2.4 Statistics

O. mykiss: The 24, 48, 72 and 96 h LC_{50} values (those concentrations causing a 50% mortality after 24, 48, 72 and 96 h exposure) were calculated with their 95% confidence limits using the moving average angle method (U.S. Environmental Protection Agency, 1985).

D. magna: The 24 and 48 h LC_{50} values (those concentrations causing immobilisation of 50% of the D. magna exposed for 24 and 48 h) were calculated with their 95% confidence limits using the moving average angle method (U.S. Environmental Protection Agency, 1985).

S. capricornutum: Effects were evaluated by expressing chlorophyll a concentration as a percentage of mean control chlorophyll a concentration. 72 h EC_{50} values (those concentrations causing a 50% reduction in growth by comparison with mean control growth after 72 h) were calculated with their 95% confidence limits by probit analysis using log_{10}. transformed concentration values (Finney, 1971). The highest no observed effect concentration was calculated using Williams' test (Williams, 1971, 1972)

3. RESULTS

3.1 Water quality

The results of the water quality determinations are summarised below and presented in full in Appendix C.

Parameter		O. mykiss	D. magna	S. capricornutum
Temperature (°C)		16 to 17	21 to 23	21 to 24
pH		6.9 to 7.9	7.7 to 8.5	8.0 to 10.3
Dissolved oxygen	(mg/l)	7.9 to 10.4	8.0 to 9.2	N/D
Total hardness (as $CaCO_3$)	(mg/l)	254 to 268	174	N/D
Residual chlorine	(mg/l)	0.02 to 0.04	N/D	N/D

(N/D not determined)

The water quality parameters were generally in the preferred ranges for all of the tests. However the following should be noted.

The total water hardness recorded during the test with O. mykiss was above the preferred maximum of 250 mg/l ($CaCO_3$) according to the OECD guideline 203 (Fish, Acute Toxicity Test). The recorded range of 254 to 268 was not regarded sufficient to jeopardise the outcome of the study. The pH recorded during the test with S. capricornutum was within the OECD guideline 207 (Alga, Growth Inhibition Test) of ± 1 pH unit between all flasks at the start of the test. By the end of the test, 72 h later, the pH in the flasks containing the control medium and at measured concentrations of 0.24, 0.48, 0.93 and 2.0 mg/l EPIKOTE 862 had risen by more than 1 unit. This reflects good algal growth and is not regarded as sufficient to jeopardise the outcome of the study.

3.2 Toxicity tests

3.2.1 Analysis of aquatic toxicity test media

The results of the analysis of the aquatic toxicity test media are presented in Appendix D.

Figure D1, shows HPLC chromatograms of a standard solution of EPIKOTE 862 and of extracts from fresh and aged saturated solutions. The ageing of solutions appeared to provide shorter retention time, probably more water soluble material, probably alcohols formed by hydrolytic opening of the opoxide groups. These were not present to any significant extent in the EPIKOTE 862 standard. Because they were produced from the major components of the test substance, the concentration of these shorter retention time components would be a function of the amounts of the major components of EPIKOTE 862 which dissolved, and not of the loading rate used to prepare the water accommodation fraction. For that reason, dilution of a saturated solution was the appropriate experimental technique to use for this study.

For the test with O. mykiss, dilution of the saturated solution of EPIKOTE 862 prepared at 1000 mg/l resulted in a series of mean measured concentrations ranging from 4.4 to 0.1 mg/l at the start of the 24 h exposure periods. At the start of the first exposure period, the mean measured concentrations were about 50% of the mean measured concentrations at the start of the subsequent three exposure periods. The reasons for this were not investigated further. By the end of each exposure period measured concentrations fell by an average of 11 to 25% of the initial measured concentration.

For the test with D. magna, the saturated solution of EPIKOTE 862, prepared at 1000 mg/l, and the dilutions, resulted in a series of measured concentrations ranging from 7.4 to 0.24 mg/l at the start of the 48 h exposure period. The fall in concentrations over the 48 h exposure period ranged from 8 to 26% of the initial measured values.

For the test with S. capricornutum, the saturated solution of EPIKOTE 862, prepared at 1000 mg/l, and the dilutions, resulted in a series of measured concentrations ranging from 10 to 0.31 mg/l at the start of the 72 h exposure period. The fall in concentrations over the 72 h exposure period ranged from 34% to 44% of the initial measured value.

The results of the toxicity tests are expressed in relation to measured concentrations of EPIKOTE 862 in the test media.

3.2.2 Oncorhynchus mykiss

The results of the toxicity test with O. mykiss are given in Table 1. Figure 1 is a plot of mortality of O. mykiss after 96 h exposure to a range of measured concentrations of EPIKOTE 862.

All of the O. mykiss died after 4 h exposure to 4.0 mg/l of EPIKOTE 862. By the end of the test all of the O. mykiss had died at 4.0, 1.2 and 0.97 mg/l and 50% had died at 0.55 mg/l. There were no mortalities or sublethal effects of EPIKOTE 862 to O. mykiss at 0.24 and 0.16 mg/l.

The LC_{50} values, calculated on the basis of mean determined concentrations of EPIKOTE 962 in the test media together with their 95% confidence intervals are summarised below

	LC_{50} (mg/l)	95% Confidence interval (mg/l)
24 h	1.1	0.99-1.28
48 h	0.73	0.58-0.93
72 h	0.55	0.41-0.69
96 h	0.55	0.41-0.69

3.2.3 Daphnia magna

The results of the toxicity test with D. magna are given in Table 2. Figure 2 is a plot of the percentage of D. magna immobilised after 48 h exposure to a range of mean measured concentrations of EPIKOTE 862

After 48 h exposure to EPIKOTE 862 all of the D. magna were immobilised at 6.6 and 3.4 mg/l. At 1.6 mg/l of EPIKOTE 862, 55% of the D. magna were immobilised by the end of the 48 h exposure period.

The 24 and 48 h EC_{50} values, calculated on the basis of mean determined concentrations of EPIKOTE 862 in the test media over the duration of the test were 3.2 mg/l and 1.6 mg/l respectively (95% confidence intervals 2.4 to 4.7 mg/l and 1.2 to 2.2 mg/l respectively).

3.2.4 Selenastrum capricornutum

The results of the toxicity test with S. capricornutum are given in Table 3. Figure 3 is a plot of the measured concentration of EPIKOTE 862 versus growth inhibition measured by the concentration of chlorophyll a in the test cultures at the end of the test. Based on the reduction in chlorophyll a relative to controls EPIKOTE 862 in the test media, the 72 h EC_{50} to S. capricornutum was calculated to be 1.8 mg/l (95% confidence intervals 1.5 to 2.1 mg/l).

The highest no observed effect concentration (NOEC) of EPIKOTE 862 to S. capricornutum was calculated to be 0.24 mg/l.

Signed: Louis Jr (Study Director)

Date: 1/07/93.

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Table 1 - Toxic symptoms exhibited by O. mykiss exposed to a range of concentrations of EPIKOTE 862

Mean measured concentration of EPIKOTE 862 (mg/l)	Number of fish	Observation time and symptom classification*																							
		3 h					24 h					48 h					72 h					96 h			
		a	b	c	d	e	a	b	c	d	e	a	b	c	d	e	a	b	c	d	e	a	b	c	d	e
Control	10	10					10					10					10					10				
0.16	10	10					10					10					10					10				
0.24	10	10					10					10					10					10				
0.55	10	10					10					10					5			5		5				5
0.97	10	10						9	1								10				10					10
1.2	10	10									10						10				10					10
4.0	10			6	4						10						10				10					10

* Symptoms classification

(a) Number of fish exhibiting no toxic symptoms.
(b) Number of fish swimming normally but exhibiting toxic symptoms e.g. increased cough frequency, hyperventilation.
(c) Number of fish swimming abnormally e.g. on side or back.
(d) Number of fish immobilised e.g. lying on bottom of tank or floating at surface, but still alive. Fish in this category were removed from the vessels and their numbers added to the total in category 'e' at subsequent observations.
(e) Number of fish dead.

Table 2 - Immobilisation of D. magna exposed to a range of concentrations of EPIKOTE 862

Mean measured concentration of EPIKOTE 862 (mg/l)	Number of D. magna	Number immobilised	
		24 h	48 h
Control	10	0	0
	10	0	0
0.23	10	0	0
	10	0	0
0.4	10	0	0
	10	0	0
0.79	10	0	0
	10	0	0
1.6	10	0	5
	10	0	6
3.4	10	9	10
	10	3	10
6.6	10	10	10

Table 3 - Growth of S. capricornutum cultures exposed to a range of concentrations of EPIKOTE 862

Mean measured concentration of EPIKOTE 862 (mg/l)	Chlorophyll a concentration at t = 72 h (µg/l)	Mean chlorophyll a concentration (µg/l)	Chlorophyll a concentration as a % of controls	Mean percent reduction in chlorophyll a compared to controls
Control	130 139	128	-	-
	146 121			
	107 125			
0.24	126	115	99	10
	103		81	
	116		91	
0.48	121	103	95	19
	115		90	
	74		58	
0.93	110	99	86	22
	98		66	
	89		70	
2.0	*	61	-	52
	48		37	
	74		58	
4.2	35	35	28	72
	31		25	
	39		31	
6.3	21	13	17	89
	9		7	
	10		8	

* Sample mistakenly discarded.

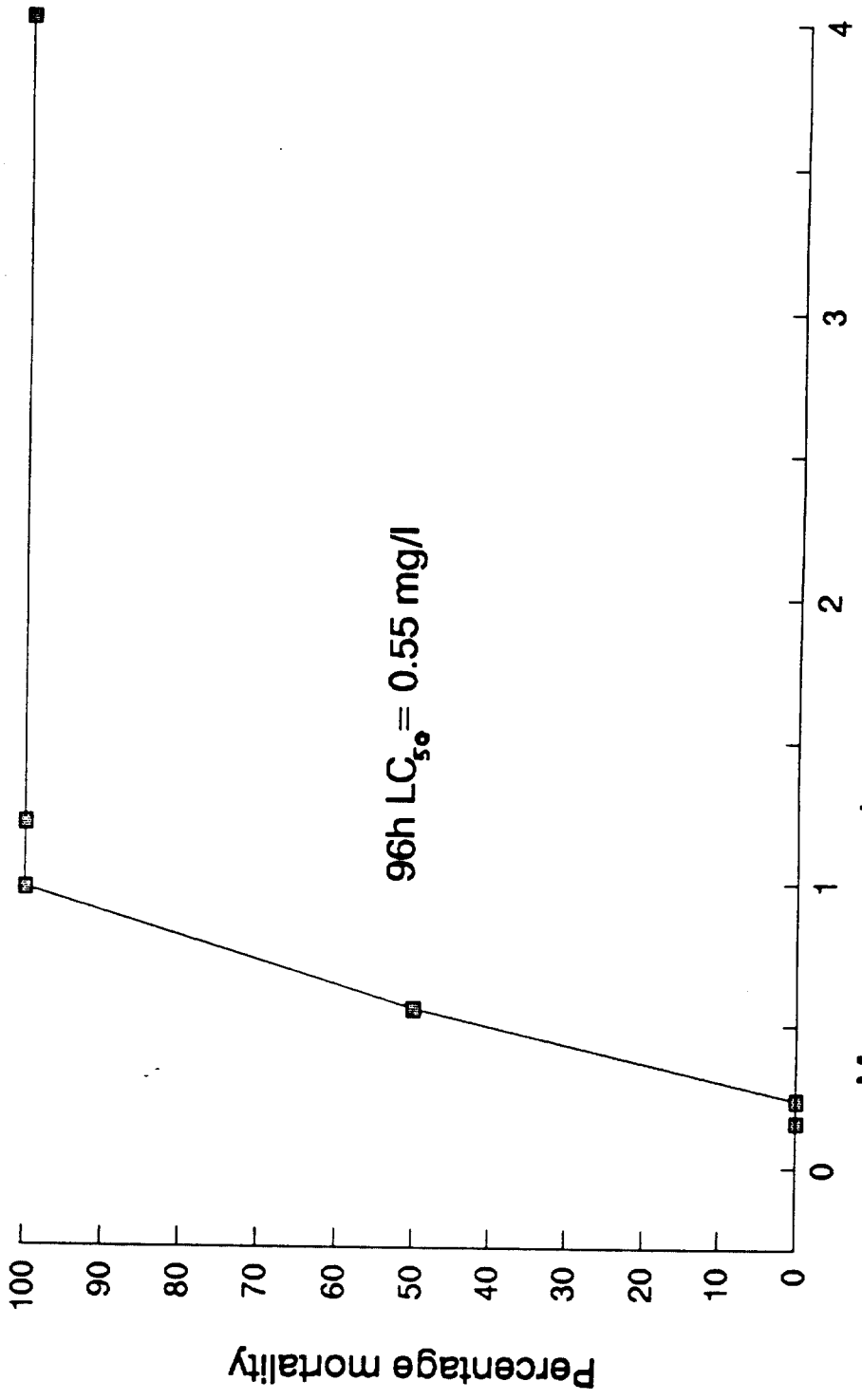


Figure 1. Percentage mortality of O. mykiss after 96h exposure to a range of concentrations of EPIKOTE 862.

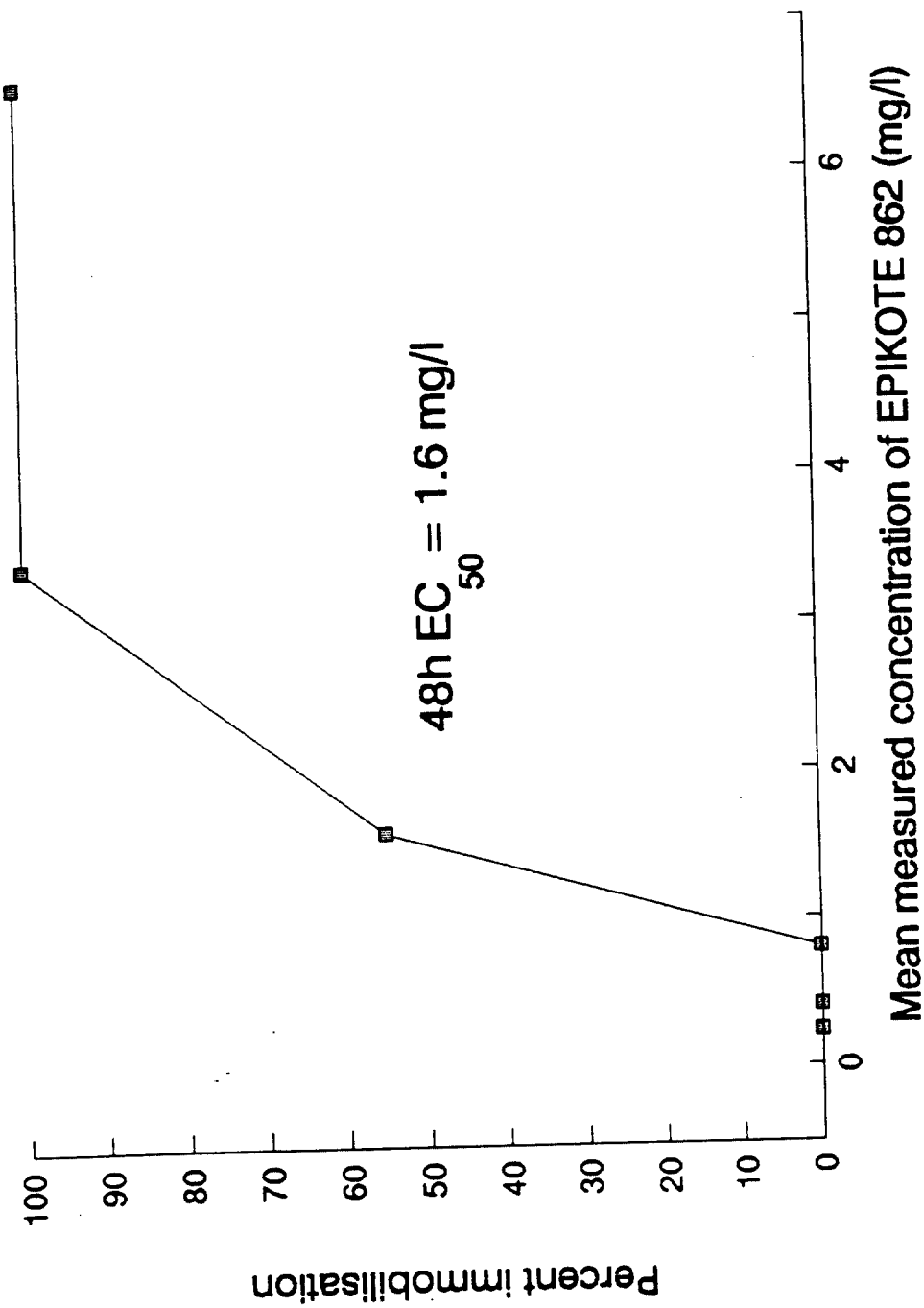


Figure 2. Percentage immobilisation of D. magna after 48h exposure to a range of concentrations of EPIKOTE 862.

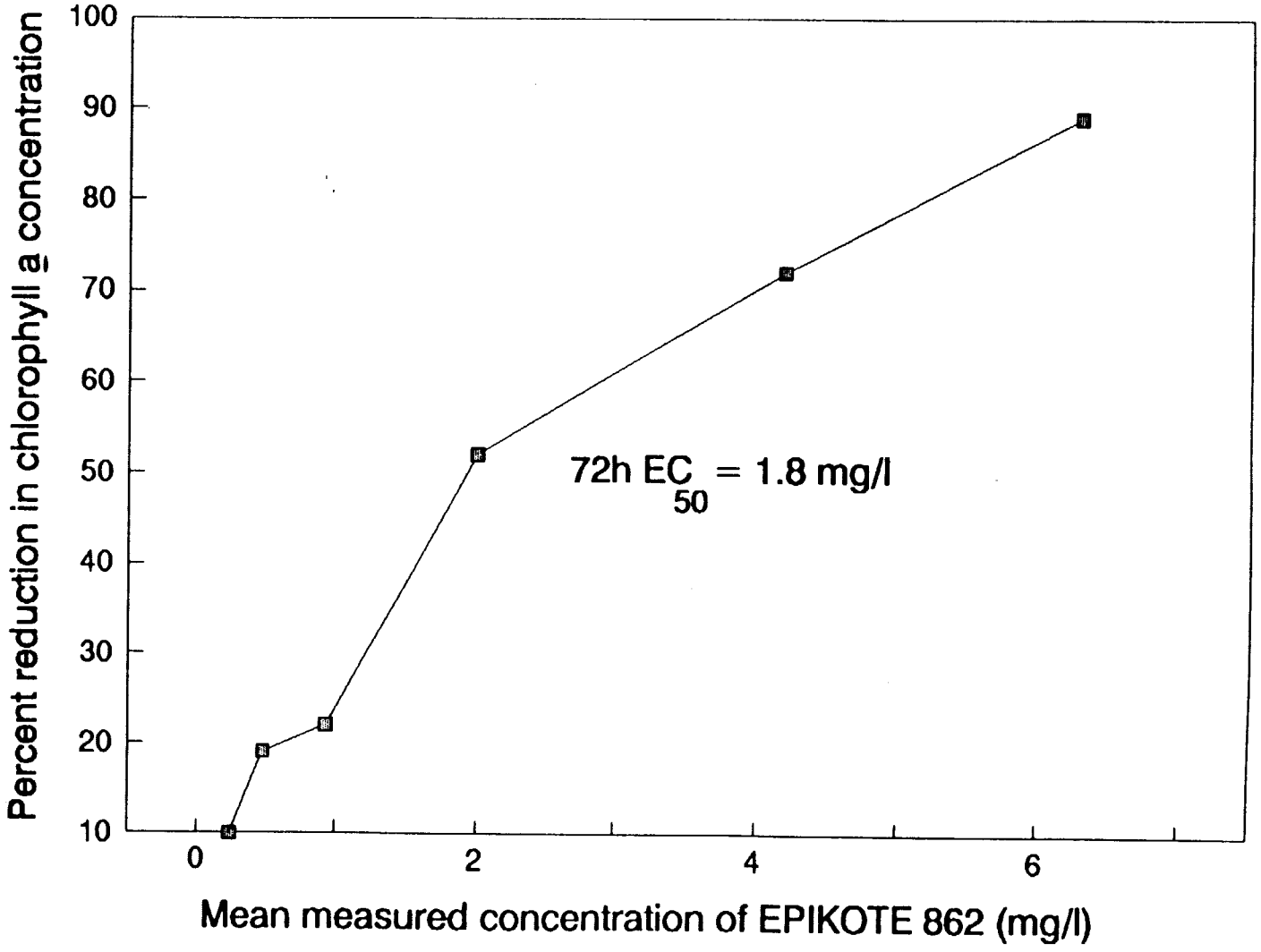


Figure 3. Percentage reduction in chlorophyll a of S.capricomutum relative to controls after 72h exposure to a range of concentrations of EPIKOTE 862.

APPENDIX A

Title: COMPOUND CONTROL REPORT (Expt. Number 4607)

Test substance

Identity of the test substance

The data for the test substance released for use in this experiment are tabulated below.

NAME	EPIKOTE 862
CODE NUMBER	L1262
BATCH (& OTHER) NUMBERS	Tank 1126; Indent 9450/9909
TOXICOLOGY REF. NUMBER	ST92/100
SOURCE	Shell Nederland Chemie B.V., Pernis
DATE RECEIVED	31st March 1992
APPEARANCE	Clear colourless viscous liquid
CHARACTERIZATION	**Actual analysis**

Viscosity @ 25°C	3.51 Pa.s	(ASTM D 445)
Epoxy group content	5900 mmol/kg	(SMS 2026)
Colour (Pt/co)	45	(ISO 2211)

Ref. Certificate of analysis dated 24-03-92 supplied by Shell Nederland Chemie B.V.

No claim of GLP compliance is made in respect of these data.

DATE RELEASED	9th April 1992

Storage of this test substance

Following its arrival in Compound Control this test substance was stored in the dark at ambient temperature.

Stability of this test substance

The data sheet (EK1.1.102) supplied with this sample stated that, provided EPIKOTE 862 was stored at normal temperatures in such a way that moisture is excluded, the storage life should be at least one year. The storage conditions employed complied with this provision, and therefore I consider that the test substance was stable for the duration of this study.

Responsible Practitioner _____ (signature)

30 October 1992 (date)

APPENDIX B

Title: MAINTENANCE/CULTURE OF TEST ORGANISMS (Expt. Number 4607)

The test organisms used in this experiment were maintained and cultured as described below.

1. Procedure for the maintenance of Oncorhynchus mykiss Walbaum

O. mykiss for use in toxicity tests are obtained from commercial hatcheries. On arrival at the laboratory the fish are given a batch number and are inspected for signs of disease. The fish are placed in ~300 l, circular, self-cleaning, fibre-glass tanks at a density appropriate to their size. The tanks receive a continuous flow of temperature controlled water of a suitable and defined quality (Appendix C). The fish are fed a maintenance ration of Mainstream Trout Food No. 03 (BP Nutrition Ltd.), the quantities fed depending upon the size and age of the fish and the temperature of the water. Fish mortalities and dissolved oxygen concentration in the tanks are recorded daily. The fish are held in a temperature controlled room, 13-17°C, under artificial light with a 16 h light 8 h dark cycle. The fish are deemed acceptable for testing if the cumulative mortality in the batch is less than 5% over a 7 day period preceding the test. The stock is not used for testing if mortality exceeds 5% or if disease is apparent.

After a 48 h settling-in period, the fish are acclimated to the test conditions for a minimum of 7 days before a test begins. They are not fed in the 24 h preceding a test. At the conclusion of each test a sample of ten fish used in the test are weighed (wet weight) and measured (fork length).

2. Procedure for the culture of Daphnia magna (Straus)

The laboratory culture of D. magna is derived from a clone held at ICI Brixham Laboratory. The ICI clone was itself obtained from the Institut National de Recherche Chimique Applique (I.R.Ch.A.), France.

For the first 14 days the D. magna are cultured in 1 litre pyrex glass beakers containing 0.8 litre of reconstituted freshwater (Appendix C). From Day 15 onwards they are cultured in a 2 litre beaker containing 1.5 litre of reconstituted freshwater. Each vessel and its contents are referred to as a 'culture'. New cultures are started with animals less than 24 h old, at a density of about 12 per litre. The cultures are held in a temperature controlled room, nominally 18-22°C, under artificial light in a 16 h light 8 h dark cycle. The cultures are fed daily with a concentrated suspension of Chlorella vulgaris to give a concentration of approximately 0.10×10^6 cells/ml. The C. vulgaris is obtained from a 5 litre culture grown under semi-axenic conditions. Prior to use the C. vulgaris is concentrated to about $100\text{-}200 \times 10^6$ cells/ml by filtration and resuspension in reconstituted freshwater (Appendix C). The C. vulgaris is stored in a refrigerator at 4°C and used within one week.

The water in the culture vessels is renewed twice weekly. Young are removed daily using a pipette (if not required for testing) or sieve (56 mesh/cm). Cultures are discarded when 28 days old.

Young for use in acute toxicity tests are collected from the third brood onwards of cultures aged between 15 and 28 days. 24 h before a test is set up, any young present in the cultures are removed and discarded. Approximately 23 h later, young for use in the test are removed from the parent culture vessel and transferred to fresh culture medium. The young are then left for one hour before selecting actively swimming individuals for use in the test.

Young for testing are not taken from cultures which contain adults bearing ephippia; these cultures are discarded.

3. Procedure for the culture of Selenastrum capricornutum (Prinz)

Axenic stock cultures of S. capricornutum are maintained on agar plates. These are used to inoculate liquid cultures which, while in exponential phase growth, are in turn used to inoculate test solutions.

Source

The axenic strain of S. capricornutum (ATCC 22662) was obtained from the American Type Culture Collection, Maryland, Ohio, U.S.A.

Maintenance of cultures

Agar plates are prepared by adding 1.5% mass/volume agar to liquid medium (Appendix C) prior to autoclaving. On cooling the agarified medium is poured into 9 cm diameter sterile plastic petri dishes and allowed to set. The algal cultures are streaked onto these plates and maintained under continuous illumination at 18-22°C in a Gallenkamp vertical incubator. The cultures are renewed approximately every fortnight.

Cultures in liquid medium (Appendix C) are initiated with cells transferred on a sterile loop from an agar plate. The cultures are grown as 100 ml batch cultures in 250 ml Erlenmeyer flasks for up to 4 days. The cultures are maintained at 22-26°C in a Gallenkamp orbital incubator under constant illumination (~ 3000 lux).

Inoculation of test cultures

The quantity of inoculum introduced into each test vessel is sufficient to give a concentration of 5000 cells ml/l. The cell concentration of an exponentially growing stock culture is measured using a Coulter Multisizer and the required inoculum volume calculated. All flasks used for a particular test are inoculated from the same stock culture.

APPENDIX C

(Expt. Number 4607)

Title: WATER QUALITY

INTRODUCTION

The quality of the medium in which organisms are held, prior to and during toxicity tests, can influence the results of the tests. This report gives details of the quality of the media used for acclimation/culture, and for testing, in the experiments described in the main report.

MATERIALS AND METHODS

Source and treatment

Oncorhynchus mykiss

The water used for maintenance of stocks and for the toxicity test with O. mykiss is the laboratory mains supply. The water comes from two pumping stations (Highstead and Eastling) controlled by the Mid Kent Water Company. The water is obtained from bore holes in the chalk of the North Downs. The only chemical treatment prior to its arrival in the laboratory is chlorination to 0.1 mg/l.

In the laboratory the water is filtered (PALL MDY 1001 Y400) to remove all particles larger than 15 μm (90% of particles greater than 10 μm) and passed through activated carbon filters (Cano model CT) to remove chlorine and organic contaminants. Both particle and activated carbon filter cartridges are renewed as recommended by the manufacturers. Heat exchange units (stainless steel and perspex) are used to adjust the temperature of the water. Prior to use in tests the water is vigorously aerated for several hours to ensure that no residual chlorine remains.

Samples of the water, after filtration but before aeration, are taken at approximately 6 month intervals and analysed for a number of parameters (Table C.1). These analyses are carried out by Mid Kent Scientific Services Limited.

Daphnia magna

The water used for the culturing and testing of D. magna is a reconstituted fresh water prepared by dissolving the following amounts of Analar grade salts in Millipore "Milli-Q" filtered water:-

$NaHCO_3$	192 mg/l
$CaSO_4.2H_2O$	120 mg/l
$MgSO_4.7H_2O$	240 mg/l
KCl	8 mg/l

This recipe has been recommended as one suitable for producing a 'hard' water by the U.S. Environmental Protection Agency (1975).

Before use for culturing, a soil extract is added to the reconstituted fresh water at a rate of 20 ml/l. The soil extract is prepared by autoclaving 100 g soil per litre of Millipore "Milli-Q" filtered water for 15 minutes at 120°C. Solids are removed by filtration through Whatman GF/C paper.

Selenastrum capricornutum

A nutrient medium is prepared by dissolving Analar grade salts in Millipore "Milli-Q" filtered water. Nutrient concentrations are those described by Miller and Green (1978) except that boric acid is present at 105 µg/l (184 µg/l in Miller and Green), and sodium bicarbonate at 50 mg/l (15 mg/l in Miller and Green).

The medium (excluding sodium bicarbonate) is autoclaved at 1.0 kg/cm^2 for 15 min. On cooling, 20 ml/l of a 0.45 µm millipore-sterilised solution of sodium bicarbonate (2.5 g/l) is added to yield a final concentration of 50 mg/l in the nutrient medium.

Water quality during stock culture, maintenance and acclimation

O. mykiss: The temperature of the water is monitored at hourly intervals by a computer controlled thermocouple system which outputs to a dedicated microcomputer. Temperature data are stored on the hard disc and can be retrieved either as hard copy for visual inspection and reporting or on magnetic tape for archiving. The pH, concentration of dissolved oxygen, and total hardness are checked weekly. The pH measurements are made with a calibrated pH meter. The dissolved oxygen measurements are made with a YSI 57 dissolved oxygen meter calibrated in air saturated water immediately prior to use. Total hardness is determined by titration against EDTA in the presence of ammonia buffer and a suitable indicator.

D. magna: The temperature of water in a beaker adjacent to the cultures is monitored and the pH, dissolved oxygen concentration and total hardness of each batch of culture medium used are checked, using the system and procedures described for O. mykiss.

S. capricornutum: The temperature in the incubator is monitored using the system described for O. mykiss. The pH of the media is checked prior to use with a calibrated pH meter.

Water quality during tests

O. mykiss: The water temperature in a vessel adjacent to the test aquaria was monitored as described previously. The pH and dissolved oxygen concentration was determined in the control and each test media at the start and conclusion of each exposure period. The total hardness and residual chlorine concentrations were determined for each new batch of dilution water used. Residual chlorine concentration is determined using a BDH Lovibond Nesslerizer. All other determinations are made using the methods described previously.

D. magna: The temperature of water in a vessel adjacent to the test vessels was monitored as described previously. The pH and concentration of dissolved oxygen in the control and each test media were determined at the start and conclusion of the exposure period. The total hardness of the water used in the test was determined. All determinations were made using the methods described previously.

S. capricornutum: The temperature in the orbital incubator was monitored as described previously. The pH of the control and each test media was determined at the start and conclusion of the exposure period.

RESULTS

Quality of laboratory mains supply

The most recent data set and the range of values obtained since monitoring of the laboratory water supply began are given in Table C1.

Water quality during the acclimation of O. mykiss and the culture of D. magna and S. capricornutum

The results of the determinations are given in Table C2 (O. mykiss), Table C3 (D. magna) and Table C4 (S. capricornutum).

Water quality during toxicity tests

The results of the determinations are given in Table C5 (O. mykiss), Table C6 (D. magna) and Table C7 (S. capricornutum).

REFERENCES

Miller, W. E. and Green, J. C. (1978).
The Selenastrum capricornutum (Prinz) algal bottle test.
EPA-600/9-78-018.

U.S. Environmental Protection Agency (1975).
Methods for acute toxicity testing with fish, macro-invertebrates and amphibians.
EPA-660/3-75-009.

Table C1 - Quality of laboratory mains supply, after filtration (10 μm) and passage through activated carbon.

Parameter	Overall range (12/10/89 - 26/5/92) (n = 5)	Latest value (26/5/92)	
CONDUCTIVITY	519 - 534	522	(μs/cm)
pH	7.0 - 7.5	7.4	
SAT. INDEX	-1.08 - -0.1	ND	(NO)
TOTAL SOLIDS	205 - 365	364	(mg/l)
ALKALINITY	254 - 264	264	(mg/l)
CARBON DIOXIDE	ND - ND	ND	(mg/l)
TOTAL HARDNESS	273 - 285	285	(mg/l)
PERMANGANATE VALUE	<0.04 - 0.12	ND	(mg/l)
TOTAL CHLORINE	<0.02 - 0.02	<0.02	(mg/l)
PHENOL	<0.5 - <5	<5	(μg/l)
TOTAL CALCIUM	106 - 110	110	(mg/l)
TOTAL MAGNESIUM	1.6 - 2.6	2.6	(mg/l)
TOTAL IRON	<0.01 - <0.02	<0.01	(mg/l)
TOTAL MANGANESE	<0.02 - <0.02	<0.02	(mg/l)
TOTAL MERCURY	<0.2 - <0.2	<0.2	(μg/l)
TOTAL LEAD	<5 - <5	<5	(μg/l)
TOTAL CADMIUM	<0.5 - <0.5	<0.5	(μg/l)
TOTAL ARSENIC	<5 - <5	<5	(μg/l)
TOTAL COPPER	<0.02 - 0.09	<0.02	(mg/l)
TOTAL ZINC	<0.02 - 0.03	0.03	(mg/l)
TOTAL SODIUM	10 - 11	10	(mg/l)
TOTAL POTASSIUM	1.0 - 1.4	1.1	(mg/l)
CHLORIDE	17 - 19	19	(mg/l)
FLUORIDE	0.09 - 0.10	0.09	(mg/l)
NITRATE	3.9 - 4.0	4.0	(mg/l)
NITRITE	<1 - 4	<1	(μg/l)
ORTHO PHOSPHATE	0.03 - 0.04	0.04	(mg/l)
TOTAL SILICA	9.6 - 11.4	9.6	(mg/l)
SULPHATE	4 - 6	6	(mg/l)
AMMONIACAL N	<0.002 - <0.01	<0.01	(mg/l)
ALBUMINOID N	<0.002 - <0.002	ND	(mg/l)

ND = not determined

Table C2 - Water quality during the acclimation of the O. mykiss i.e. two most recent sets of readings prior to test

Date	24.8.92	2.9.92
Temperature (°C) [monitored at hourly intervals]	15 - 17	
Total water hardness (mg/l as $CaCO_3$)	272	178
pH	7.8	7.3
Concentration of dissolved oxygen (mg/l)	9.8	9.8

Table C3 - Water quality during the culture of the parents of the D. magna used in the test

Date	23.6.92	26.6.92
Temperature (°C) [monitored hourly intervals]	19 - 20	
Total water hardness (mg/l as $CaCO_3$)	170 - 176	
pH	8.1 - 8.1	
Concentration of dissolved oxygen (mg/l)	10.4 - 10.6	

Table C4 - Media quality during the growth of the S. capricornutum starter culture

Nominal temperature range (°C)	21 - 23
pH prior to use	9.7

Table C5 - Water quality during the test with O. mykiss

Time (h)		0	24	48	72	96
Solution tested		Fresh	Old/Fresh	Old/Fresh	Old/Fresh	Old
Temperature (°C) [Monitored at hourly intervals]		16 - 17				
Total hardness (mg/l as $CaCO_3$)		254	- /260	- / 268	- / 256	-
pH	Control	6.9	7.7/7.3	7.6/ 7.4	7.6/ 7.5	7.7
	31.2 mg/l	7.4	7.6/7.3	7.3/ 7.5	7.4/ 7.6	7.8
	62.5 mg/l	7.4	7.2/7.4	7.4/ 7.5	7.7/ 7.5	7.9
	125 mg/l	7.3	7.7/7.3	7.6/ 7.5	7.8/ 7.5	7.9
	250 mg/l	7.3	7.6/7.4	7.7/ -	- / -	-
	500 mg/l	7.3	7.7/ -	- / -	- / -	-
	1000 mg/l	7.3	7.8/ -	- / -	- / -	-
Conc. of dissolved oxygen (mg/l)	Control	10.4	8.9/10.2	8.7/10.0	8.6/ 9.8	8.7
	31.2 mg/l	10.2	8.7/10.2	8.5/ 9.8	8.3/ 9.9	8.3
	62.5 mg/	10.2	8.4/ 9.9	8.3/ 9.7	8.4/ 9.9	7.9
	125 mg/l	10.4	8.2/10.2	8.3/ 9.9	8.7/ 9.9	8.2
	250 mg/l	10.2	8.0/10.4	8.2/ -	- / -	-
	500 mg/l	9.9	8.9/ -	- / -	- / -	-
	1000 mg/l	9.8	9.2/ -	- / -	- / -	-
Residual chlorine conc. (mg/l)		0.04	- /0.04	- / 0.04	- / 0.02	-

Table C6 - Water quality during the test with D. magna

Time (h)		0	48
Temperature (°C) [Monitored at hourly intervals]		21 - 23	
Total hardness (mg/l as $CaCO_3$)		174	
pH	Control	8.2	8.4
	0.23 mg/l	7.7	8.5
	0.4 mg/l	7.8	8.5
	0.79 mg/l	8.1	8.5
	1.6 mg/l	8.2	8.5
	3.4 mg/l	8.2	8.5
	6.6 mg/l	8.3	8.5
Conc. of dissolved oxygen (mg/l)	Control	9.2	9.0
	0.23 mg/l	8.7	8.2
	0.4 mg/l	8.4	8.0
	0.79 mg/l	8.7	8.3
	1.6 mg/l	8.8	8.4
	3.4 mg/l	8.6	8.2
	6.6 mg/l	8.4	8.0

Table C7 - Temperature and pH during the test with S. capricornutum

Time (h)		0	72
Temperature(°C)[Monitored at hourly intervals]		21 - 24	
pH	Control	8.0	10.1
	0.24 mg/l	8.6	10.1
	0.48 mg/l	8.6	10.3
	0.93 mg/l	8.3	10.2
	2.0 mg/l	8.3	9.8
	4.2 mg/l	8.3	9.2
	6.3 mg/l	8.3	8.7

Title of Main Report: EPIKOTE 862: acute toxicity to Oncorhynchus mykiss, Daphnia magna and Selenastrum capricornutum

Experiment Number: 4607

APPENDIX D

Author: D.D. Lad

Scientific Reviewer: M.K. Baldwin

Title of Appendix: Chemical analysis of test media

Summary: An HPLC procedure for the analysis of the test media for Epikote 862 is described, and the results of the analyses are reported.

D. D. Lad 1/07/93.
(Signature) (Date)

1. INTRODUCTION

A high-performance liquid chromatographic (HPLC) method was developed to determine the concentration of EPIKOTE 862 in water. The substance is extracted from the water using a Whatman ODS-2 octadecyl solid phase extraction cartridge, then analysed by HPLC using a reversed phase column with ultraviolet absorption detection. Calibration is by external standardisation with solutions of EPIKOTE 862. The method is shown as Attachment D1. The results of application of this method to the analysis of aqueous test media from toxicity tests with Oncorhynchus mykiss, Daphnia magna and Selenastrum capricornutum are reported. The test media were saturated solutions of EPIKOTE 862 or dilutions thereof. The highest concentration test medium was produced by mixing 1000 mg of the test substance per litre of untreated medium, then using the aqueous phase. The lower concentrations were produced by diluting the highest concentration medium with untreated medium.

2. EXPERIMENTAL

a. Sampling and analysis programme for the test media

O. mykiss

The test media samples from the O. mykiss tests were prepared freshly each day for the duration of the test, which began on 7th September and finished on 11th September 1992. On each occasion a single sample of about 250 ml was taken from the fresh and the old media with application rates of 0, 31.25, 62.5, 125, 250, and 500 mg/l, except where certain application rates had been discontinued.

The test media samples from the D. magna and S. capricornutum tests were taken at the start and at the end of the exposure periods. Samples for analysis from these tests were received as follows:-

D. magna

30th June 1992, start of study (T = 0), one sample each from application rates of 0, 31.25, 62.5, 125, 250, 500, and 1000 mg/l.

2nd July 1992, end of study, (T = 48 hours), one sample each from application rates of 0, 31.25, 62.5, 125, 250, 500, and 1000 mg/l.

S. capricornutum

7th July 1992, start of study (T = 0), one sample each from application rates of 0, 31.25, 62.5, 125, 250, 500, and 1000 mg/l.

10th July 1992, end of study (T = 72 hours), one sample each from application rates of 0, 31.25, 62.5, 125, 250, 500, and 1000 mg/l.

Note:- A portion of the control medium taken at each sampling time was used for the determination of the percentage recovery during the analysis procedure.

All samples were analysed on the day of receipt.

b. Analysis recovery determinations performed with test media analyses

Analysis recovery determinations were performed by adding, at the time of analysis, a known amount of the test substance in acetonitrile to a sample of control medium.

3. RESULTS

a. Results of analysis of samples from the aquatic toxicity studies

The HPLC chromatogram of EPIKOTE 862 is complex, with at least nine components being visible. Four of them are large compared with the rest, and the concentrations of EPIKOTE 862 in samples are measured using those four peaks. It is assumed that the extinction coefficients for UV light at 230 nm are the same for equal weights of the four major components. That is a reasonable assumption for an approximation, since the absorptions are caused, predominantly, by the aromatic rings in the substance, and they will not be influenced much by isomerism. The larger molecular weight components will have more aromatic rings in them, because they contain more diphenylol methane units, so the absorption will rise as the molecular weight increases. A series of typical chromatograms is shown as Figure D1. Chromatograms from saturated solutions, especially aged ones, showed early peaks which were absent or at low abundance in the standard solutions. These were not included in quantitative measurements. The results of analysis of the test media for EPIKOTE 862 are shown in Tables D1, D2, and D3. They have been rounded up to two significant figures. Analytical recovery data are shown in Table D4, for which the addition concentrations were from 0.2 to 2.0 mg/litre. The percentage recoveries ranged from 109% to 90%.

For the test with O. mykiss, dilution of the saturated solution of EPIKOTE 862 prepared at 1000 mg/litre resulted in a series of mean measured concentrations ranging from 4.4 to 0.1 mg/litre at the start of each of the 24 h exposure periods.

At the start of the first exposure period, the mean measured concentrations of the dilutions, which ranged from 125 to 15.6 mg/litre, were about 50% of the mean measured concentrations at the start of the subsequent three exposure periods. The reasons for this were not investigated further.

By the end of each exposure period, measured concentrations fell by an average of 11% to 25% of the initial measured concentration.

For the test with D. magna, the saturated solution of EPIKOTE 862, prepared at 1000 mg/litre and then diluted, resulted in a series of measured concentrations ranging from 7.4 to 0.24 mg/litre at the start of the 48 h exposure period. The fall in concentration over the 48 h exposure period ranged from 8% to 26% of the initial measured value.

For the test with S. capricornutum, the saturated solution of EPIKOTE 862, prepared at 1000 mg/litre and then diluted, resulted in a series of measured concentrations ranging from 10 to 0.31 mg/litre at the start of the 72 h exposure period. The fall in concentration over the 72 h exposure period ranged from 34% to 44% of the initial measured value.

Table D5 illustrates the way that HPLC profiles of extracts from fresh and old saturated solutions of Epikote 862 compare with those from standard solutions of that substance. The four components measured elute from the HPLC system in the order A, B, C, D and, together, they comprise about 88% of the total peak area. Comparing the relative areas of the four peaks for the standard solutions with those from the extracts obtained during the D. magna test media, which was chosen as being representative of all of the test media used in this study, there was little change in peak A, the size of peak B had increased by about 10%, and peak C had increased by about 30%. The relative size of peak D had approximately halved. The components eluting later than peak D, which were not quantified, were much less prominent in the extracts from the samples than in the standard solutions. These differences are probably a reflection of the different water affinities of the various components of Epikote 862. There were no major differences between the profiles obtained with extracts from fresh and old saturated solutions.

Table D1 - Results of analysis of test media for EPIKOTE 862 during the O. mykiss test

Nominal concentrations of EPIKOTE 862 (mg/l)	Concentration of EPIKOTE 862 (mg/l)								Geometric mean concentration				
	Day 0	Day 1		Day 2		Day 3		Day 4					
	Fresh	Old	Fresh	Old	Fresh	Old	Fresh	Old	Day 1	Day 2	Day 3	Day 4	Overall
0 (control)	<0.01	0.03	0.01	<0.01	<0.01	<0.01	<0.01	<0.01	-	<0.01	<0.01	<0.01	(<0.01)
15.6	0.10	0.12	0.22	0.20	0.16	0.16	0.19	0.16	0.11	0.21	0.16	0.17	0.16
31.25	0.16	0.15	0.32	0.22	0.31	0.21	0.36	0.26	0.16	0.27	0.26	0.30	0.24
62.5	0.39	0.27	0.66	0.47	0.73	0.78	0.76	0.53	0.32	0.56	0.75	0.65	0.55
125.0	0.66	0.45	1.91	1.60	-	-	-	-	0.54	1.7	-	-	0.97
250.0	1.27	1.05	-	-	-	-	-	-	1.2	-	-	-	1.2
500	4.45	3.50	-	-	-	-	-	-	4.0	-	-	-	4.0

Table D2 - Results of analysis of test media for EPIKOTE 862 during the D. magna test

Nominal concentrations of EPIKOTE 862 (mg/l)	Concentration of EPIKOTE 862 (mg/l)		Geometric mean mg/l
	Fresh medium (T=0)	End of test (T=2 days)	
0 (control)	<0.01	<0.01	<0.01
31.25	0.24	0.22	0.23
62.5	0.46	0.35	0.40
125.0	0.92	0.68	0.79
250.0	1.7	1.5	1.6
500.0	3.7	3.1	3.4
1000.0	7.4	5.9	6.6

Table D3 - Results of analysis of test media for EPIKOTE 862 during the S. capricornutum test

Nominal concentration of EPIKOTE 862 (mg/l)	Concentration of EPIKOTE 862 (mg/l) Fresh medium (T=0)	End of test (T=3 days)	Geometric mean
0 (control)	<0.01	<0.01	<0.01
31.25	0.31	0.19	0.24
62.5	0.64	0.36	0.48
125.0	1.2	0.71	0.93
250.0	2.5	1.6	2.0
500.0	5.4	3.3	4.2
1000	10	6.6	6.3

Table D4 - Results of recovery determinations with EPIKOTE 862 added to blank media

Test	Nominal concentration (mg/litre)	Recovery (percent of that added) Day 0	Day 1	Day 2	Day 3	Day 4
O. mykiss	0.2	109			104	93
O. mykiss	0.2	90			98	95
O. mykiss	2.0		93			
O. mykiss	2.0		92			
O. mykiss	2.0		95			
O. mykiss	0.5			107		
O. mykiss	0.5			105		
D. magna	2.0	99				
D. magna	2.0	99				
D. magna	1.0			100		
D. magna	1.0			101		
S. capricornutum	1.0	100			99	
S. capricornutum	1.0	98			104	

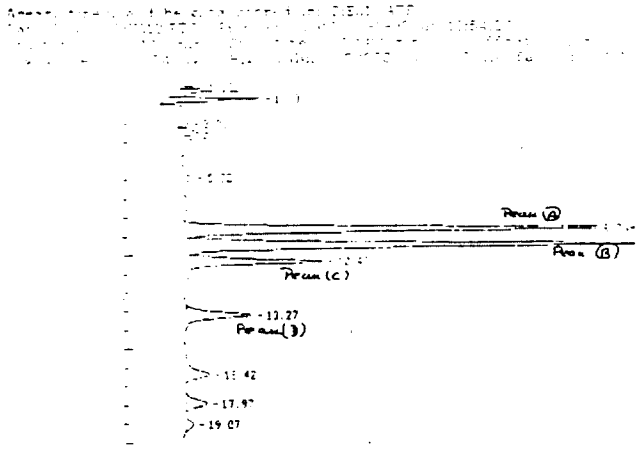
Table D5 - Chromatographic profiles of EPIKOTE 862 standards and of extracts from test media obtained during the D. magna test

Description of sample	Relative proportions of the four major HPLC peaks, assuming equal UV response (%)			
	Peak A(c)	Peak B(c)	Peak C(c)	Peak D(c)
2.5 µg/ml std. (a)	40.0	41.4	12.3	6.3
10 µg/ml std. (a)	40.4	40.9	11.9	6.8
Fresh 1 g/litre extract	37.5	44.3	15.4	2.8
Fresh 500 mg/litre extract	36.9	44.7	15.4	3.0
Fresh 125 mg/litre extract	36.9	44.3	15.6	3.2
Fresh 31.3 mg/litre extract	37.1	44.1	15.0	3.8
Old 1 g/litre extract	36.6	44.0	16.3	3.2
Old 500 mg/litre extract	36.1	45.0	16.8	2.1
Old 125 mg/litre extract	35.3	45.0	16.6	3.1
Old 31.3 mg/litre extract	33.9	45.8	17.3	3.0
2.5 µg/ml std. (b)	40.4	41.7	12.4	5.6
15 µg/ml std. (b)	40.2	40.7	12.2	6.9

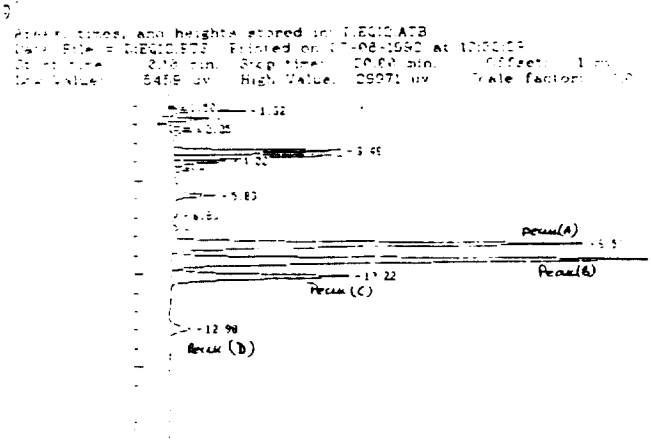
(a) = run with the fresh extracts
(b) = run with the old extracts
(c) = see figure D1 for the positions of these peaks in the chromatogram.

Figure D1 - Typical chromatograms obtained during the analysis of test media for EPIKOTE 862

(a) Standard solution containing 10 μg/ml of EPIKOTE 862



(b) Extract from freshly-prepared 125 mg/litre solution



(c) Extract from 72 hour old 125 mg/litre solution

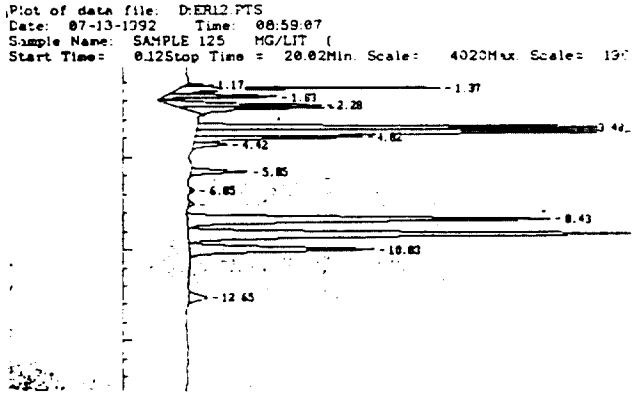
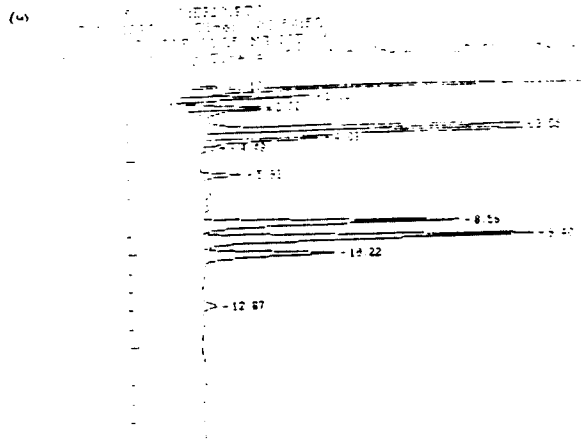
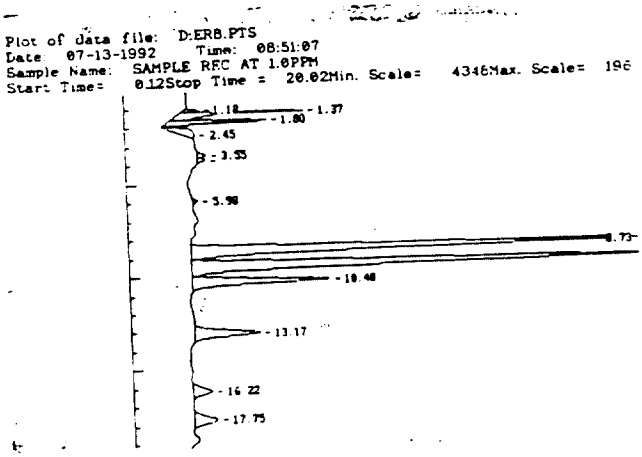


Figure D2 - Typical chromatograms obtained during the analysis of test media for EPIKOTE 862

(a) Extract from 72 hours old 31.25 mg/litre solution



(b) Extract from analysis recovery containing 1.0 mg/litre of EPIKOTE 862.



ATTACHMENT D1

METHOD FOR DETERMINING EPIKOTE 862 IN WATER

1. Summary

This method is intended for the determination of Epikote 862 in water at concentrations down to less than 0.01 ppm m/m. The lower limit of determination has not been established.

A Whatman ODS-2 octadecyl solid phase extraction cartridge, (200 mg/3 ml), is pre-washed with acetonitrile, 5 ml, followed by distilled water, 20 ml. A 100 ml portion of the aqueous sample is added to the reservoir and passed through the cartridge at about 2 ml/min, followed by distilled water, 5 ml. The eluate is discarded. The test substance is eluted from the cartridge with acetonitrile, 5 ml, which is collected, adjusted to a known volume, and analysed by HPLC.

2. Method

2.1 Sample Extraction

Because Epikote 862 will react slowly with water, the sample must be extracted immediately and not stored. For each sample, take a 3 ml ODS 2 octadecyl Whatman solid phase extraction cartridge. Elute it with 5 ml of HPLC grade acetonitrile followed by 20 ml of distilled water. Discard the eluates.

Attach a sample reservoir onto the cartridge, then add a portion of the well mixed sample of water. For samples containing nominal concentrations of 0.2 ppm or less of Epikote 862, a 100 ml portion is required. For concentrations greater than 0.2 ppm, smaller portions are used. Pass the water through the cartridge at about 2 ml/min, then pass through 5 ml of distilled water. Discard the eluate. Add HPLC grade acetonitrile, 5 ml, to the reservoir, and pass it through the cartridge at about 2 ml/min. Collect the eluate in a 5 ml measuring cylinder and adjust the volume as necessary to give extracts within the range of the standard solutions (see 2.3).

Along with each batch of samples, carry out the procedure with water to which an accurate amount of the test substance has been added, for recovery determination. Solutions containing approximately 0.2 mg/litre of the test substance, made by adding a standard solution of the test substance in acetonitrile to water, are convenient for the latter purpose.

2.2 HPLC Conditions

The following HPLC conditions are suitable for analysis of the sample extracts.

Column	:	250 mm by 4.4 mm ID Lichrocart Cartridge packed with Supersphere 100 RP 18.
Mobile Phase	:	60% Acetonitrile in water (v/v)
Flow	:	1.0 ml/min.
Injection Vol.	:	50 µl
Detector	:	Ultraviolet absorption at 230 nm.

Using the above conditions the chromatogram has nine peaks, out of which the first 4 major peaks are monitored to determine concentrations of Epikote 862. The retention time depends on the condition and age of the column.

2.3 Measurement procedure

Calibrate the HPLC system by injecting standard solutions of the test substance in acetonitrile. Primary standard solutions are made by dissolving the appropriate substance in acetonitrile to produce solutions containing 1.0 and 0.5 mg/ml. They are stable in the dark at 4°C, for one month. The range of standards needed will depend on the sensitivity of the HPLC equipment, but solutions containing 2.5 to 15.0 µg/ml of Epikote 862 are normally suitable. These are stable for one week when stored at 4°C in a refrigerator. When a linear response has been established using the first four peaks, inject samples and standard solutions in an interspersed manner. Measure the concentration of Epikote 862 in the extracts from samples by comparing the sizes of the four major peaks in standards with those from samples. Measurements can be on a peak to peak basis, in which case there will be a nominal concentration for Epikote 862 based on each of the four peaks, or on the total peak area for the sum of the four peaks, in which case only one concentration value is obtained. The latter method was used in this study.

Calculate the concentrations of the test substance in the original water sample by applying a correction to take account of the concentration factor produced by the method.

CONFIDENTIAL
SBGR.92.237

FURTHER DETAILS FOR DATA BASE ENTRY

INDEX TERMS:

10. CHEMICALS
15. TOXICOLOGY
16. RESEARCH & DEVELOPMENT

KEYWORDS:

EPIKOTE 862, Acute, Aquatic, Toxicity, Fish, Oncorhynchus mykiss, Invertebrate, Daphnia magna, Algae, Selenastrum capricornutum

EPIKOTE 862: Acute toxicity to Oncorhynchus mykiss, Daphnia magna and Selenastrum capricornutum

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WASHINGTON, D.C. 20460

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Health, Safety, and Environment
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EPA acknowledges the receipt of information submitted by your organization under Section 8(e) of the Toxic Substances Control Act (TSCA). For your reference, copies of the first page(s) of your submission(s) are enclosed and display the TSCA §8(e) Document Control Number (e.g., 8EHQ-00-0000) assigned by EPA to your submission(s). Please cite this number when submitting follow-up or supplemental information and refer to the reverse side of this page for "EPA Information Requests".

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EPA looks forward to continued cooperation with your organization in its ongoing efforts to evaluate and manage potential risks posed by chemicals to health and the environment.

Sincerely,

Terry R. O'Bryan

Terry R. O'Bryan
Risk Analysis Branch

Enclosure

12724 A

CECATS\TRIAGE TRACKING DBASE ENTRY FORM

CECATS DATA:
Submission # 8EHQ- 1093 -13724 SEQ A

TYPE: (INT.) SUPP FLWP

SUBMITTER NAME: Shell Oil Company

SUB. DATE: 10/08/93 OTS DATE: 10/13/93 CSRAD DATE: 10/25/93

INFORMATION REQUESTED: FLWP DATE:
0501 NO INFO REQUESTED
0502 INFO REQUESTED (TECH)
0503 INFO REQUESTED (VOL ACTIONS)
0504 INFO REQUESTED (REPORTING RATIONALE)

DISPOSITION:
(0639) REFER TO CHEMICAL SCREENING
0678 CAP NOTICE

VOLUNTARY ACTIONS:
(0401) NO ACTION REPORTED
0402 STUDIES PLANNED/UNDERWAY
0403 NOTIFICATION OF WORKER/OTHERS
0404 LABEL/MSDS CHANGES
0405 PROCESS/HANDLING CHANGES
0406 APP./USE DISCONTINUED
0407 PRODUCTION DISCONTINUED
0408 CONFIDENTIAL

CHEMICAL NAME: Epikote 862

CAS# 28064-14-4

INFORMATION TYPE:		P F C
0201	ONCO (HUMAN)	01 02 04
0202	ONCO (ANIMAL)	01 02 04
0203	CELL TRANS (IN VITRO)	01 02 04
0204	MUTA (IN VITRO)	01 02 04
0205	MUTA (IN VIVO)	01 02 04
0206	REPRO/TERATO (HUMAN)	01 02 04
0207	REPRO/TERATO (ANIMAL)	01 02 04
0208	NEURO (HUMAN)	01 02 04
0209	NEURO (ANIMAL)	01 02 04
0210	ACUTE TOX. (HUMAN)	01 02 04
0211	CHR. TOX. (HUMAN)	01 02 04
0212	ACUTE TOX. (ANIMAL)	01 02 04
0213	SUB ACUTE TOX (ANIMAL)	01 02 04
0214	SUB CHRONIC TOX (ANIMAL)	01 02 04
0215	CHRONIC TOX (ANIMAL)	01 02 04

INFORMATION TYPE:		P F C
0216	EPI/CLIN	01 02 04
0217	HUMAN EXPOS (PROD CONTAM)	01 02 04
0218	HUMAN EXPOS (ACCIDENTAL)	01 02 04
0219	HUMAN EXPOS (MONITORING)	01 02 04
(0220)	ECO/AQUA TOX	01 02 04
0221	ENV. OCCC/REL/FATE	01 02 04
0222	EMER INCI OF ENV CONTAM	01 02 04
0223	RESPONSE REQEST DELAY	01 02 04
(0224)	PROD/COMP/CHEM ID	01 02 04
0225	REPORTING RATIONALE	01 02 04
0226	CONFIDENTIAL	01 02 04
0227	ALLERG (HUMAN)	01 02 04
0228	ALLERG (ANIMAL)	01 02 04
0239	METAB/PHARMACO (ANIMAL)	01 02 04
0240	METAB/PHARMACO (HUMAN)	01 02 04

INFORMATION TYPE:		P F C
0241	IMMUNO (ANIMAL)	01 02 04
0242	IMMUNO (HUMAN)	01 02 04
0243	CHEM/PHYS PROP	01 02 04
0244	CLASTO (IN VITRO)	01 02 04
0245	CLASTO (ANIMAL)	01 02 04
0246	CLASTO (HUMAN)	01 02 04
0247	DNA DAM/REPAIR	01 02 04
0248	PROD/USE/PROC	01 02 04
0251	MSDS	01 02 04
0299	OTHER	01 02 04

TRIAGE DATA:

NON-CBI INVENTORY
(YES (CONTINUE))
NO (DROP)
DETERMINE

ONGOING REVIEW
YES (DROP/REFER)
NO (CONTINUE)
REFER:

SPECIES
Rainbow Trout
[redacted]
Daphnia
freshwater unicellular alga

TOXICOLOGICAL CONCERN:
LOW
MED
HIGH

USE:

PRODUCTION:

COMMENTS: Non-Cap

8e Init.

Shell Oil Company

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8EHQ-1093-12724

Contains No CBI

October 8, 1993

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ATTN: 8(e) Coordinator

Dear Sir:

SUBJECT: ACUTE TOXICITY OF EPIKOTE® 862 TO ONCORHYNCHUS MYKISS, DAPHNIA MAGNA, AND SELENASTRUM CAPRICORNUTUM.

The following information is submitted under TSCA 8(e).

The toxicity of EPIKOTE Resin 862 (CAS Number 28064-14-4) to rainbow trout (Oncorhynchus mykiss), freshwater zooplankter (Daphnia magna), and freshwater unicellular alga (Selenastrum capricornutum) was determined by exposure of the organisms to test media prepared from saturated solutions of the test substance. Note that the concentrations of the test substance varied with time. The reported results are:

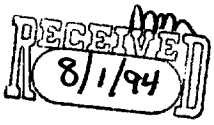
1. Oncorhynchus mykiss: The LC_{50}, 96-hours, was calculated to be 0.55 mg/liter,

2. Daphnia magna: The EC_{50}, 48 hours, was found to be 1.6 mg/liter,

3. Selenastrum capricornutum: The EC_{50}, 72 hours, was 1.8 mg/liter.

Attached is a copy of the report SBGR.92.237 entitled, "EPIKOTE 862: Acute Toxicity to Oncorhynchus mykiss, Daphnia magna and Selenastrum capricornutum.

This report is filed to provide information EPA may find useful. In no way is it intended as a waiver of any rights or privileges belonging to Shell Oil Company as the reporting corporation, its



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Very truly yours,

R. N. Shulman
General Manager
Health, Safety, and Environment
Shell Oil Company

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GROUP RESEARCH REPORT

SBGR.92.237

500 70 292 RE 9201

EPIKOTE 862: Acute toxicity to Oncorhynchus mykiss, Daphnia magna and Selenastrum capricornutum

L.E. Wyness

SICC, CMKP

SECURITY CLASS: CONFIDENTIAL

DOCUMENT TYPE: GROUP RESEARCH REPORT

DOCUMENT NUMBER: SBGR.92.237

TITLE: EPIKOTE 862: Acute toxicity to Oncorhynchus mykiss, Daphnia magna and Selenastrum capricornutum

AUTHOR(S): Wyness LE SEN/2

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Baldwin MK SPS/3

PROJECT NUMBER: SRC40593

SUB PROJECT: 4607

PROJECT TITLE: Toxicology/ecotoxicology of resins and monomers

SPONSOR: SICC, CMKP

BUDGET CODE: 500 70 292

SOURCE: Shell Research Limited, Sittingbourne Research Centre.

ORIGINATING DEPT: Environmental Research Department

DATE: July 1993

HS/WS4/502

EPIKOTE* 862: Acute toxicity to Oncorhynchus mykiss, Daphnia magna and Selenastrum capricornutum

(Experiment Number 4607)

SUMMARY:

The acute toxicity of saturated solutions of EPIKOTE 862 and dilutions of it has been determined to the fish Oncorhynchus mykiss (rainbow trout), the crustacean zooplankter, Daphnia magna and the unicellular alga, Selenastrum capricornutum.

Saturated solutions of EPIKOTE 862 were prepared by mixing EPIKOTE 862 with dilution water/culture medium for 24 h, after which the contents were allowed to settle and the aqueous phase drawn off and either used or further diluted before use in the toxicity test. For all of the toxicity tests, saturated solutions of EPIKOTE 862 were prepared at 1000 mg/l.

For the test with O. mykiss, dilutions of the saturated solution of EPIKOTE 862 resulted in a series of mean measured concentrations from 4.4 to 0.1 mg/l. By the end of each exposure period the concentrations fell by an average of 11 to 25% of the initial measured concentrations.

For the test with D. magna, dilutions of the saturated solution of EPIKOTE 862 resulted in a series of initial measured concentrations from 7.4 to 0.24 mg/l. By the end of the 48 h exposure period the concentrations fell by 8 to 26% of the initial measured concentrations.

For the test with S. capricornutum, dilutions of the saturated solution of EPIKOTE 762 resulted in a series of measured concentrations from 10 to 0.31 mg/l. By the end of the 72 h exposure period the concentrations fell by 34 to 44% of the initial measured concentrations.

The acute toxicity of EPIKOTE 862 to O. mykiss was determined in a 96 h semi-static, sealed toxicity test with daily renewal of the test media. Based upon mean measured concentrations of EPIKOTE 862 in the test media over the relevant exposure periods the 24, 48, 72 and 96 h LC_{50} values have been calculated to be respectively 1.1, 0.73, 0.55 and 0.55 mg/l.

The acute toxicity of EPIKOTE 862 to D. magna was determined in a 48 h sealed static toxicity test without renewal of the test media. Based upon mean measured concentrations of EPIKOTE 862 in the test media over the duration of the test the 24 and 48 h EC_{50} values have been calculated to be respectively 3.2 and 1.6 mg/l.

* Shell trade name

The toxicity of EPIKOTE 862 to S. capricornutum was determined in a 72 h sealed growth inhibition test in which the test medium was not renewed. Based upon mean measured concentrations of EPIKOTE 862 in the test media over the duration of the test and an analysis of the concentration of chlorophyll a in the test media at the end of the test the 72 h EC_{50} has been calculated to be 1.8 mg/l. The NOEC of EPIKOTE 862 to S. capricornutum was calculated to be 0.24 mg/l.

C. L. Volger

pp H.C. Volger Ph.D., Manager & Director Research,
Shell Research Limited,
Shell Research Limited,
Sittingbourne Research Centre,
Sittingbourne, Kent, ME9 8AG, England.

Date: 5/7/93

[redacted]

6

TEXT:

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1. INTRODUCTION

The acute toxicity of EPIKOTE 862 to the fish, Oncorhynchus mykiss, (rainbow trout) the crustacean zooplankter, Daphnia magna, and the planktonic alga Selenastrum capricornutum was determined. These data were collected within the framework of the assessment of hazard associated with the manufacture, transport, use and disposal of EPIKOTE 862.

2. MATERIALS AND METHODS

2.1 Sample

The sample of EPIKOTE 862 was received from Shell Nederland Chemie B.V. and was given the laboratory reference number ST92/100 (Appendix A).

2.2 Toxicity tests

2.2.1 Test organisms

Oncorhynchus mykiss Walbaum

Healthy O. mykiss fingerlings were obtained from Zeals Trout Farm, Wiltshire, (Batch No. RT/117) and acclimated to the test conditions for more than nine days before use (Appendix B; Appendix C). A sample of ten of the fish used in the test had a mean length of 2.9 cm (range 2.8 to 3.2 cm) and a mean weight of 0.24 g (range 0.16 to 0.34 g). The size of the fish were below the size range recommended in the OECD guideline 203 5 ± 1 cm (fish acute toxicity test). However, the difference between the recommended and actual size would not have affected the outcome of the test.

Daphnia magna (Straus)

D. magna, less than 24 h old, were taken from a laboratory culture. The culture is derived from a strain obtained (via ICI Brixham Laboratory) from I.R.Ch.A., France (Appendix B; Appendix C).

Selenastrum capricornutum (Prinz)

S. capricornutum were taken from a semi-axenic laboratory culture. The culture is derived from a strain (ATCC 22662) obtained from the American Type Culture Collection, Maryland, Ohio, U.S.A. (Appendix B; Appendix C).

2.2.2 Preparation of test media

EPIKOTE 862 was not wholly soluble in water and did not form a homogeneous and stable dispersion on contact with water. Saturated solutions of EPIKOTE 862 were prepared by mixing 1000 mg/l EPIKOTE 862 with dilution water/culture medium appropriate to each test (Appendix C) for 24 h in sealed vessels with minimum headspace. As a result of the components of EPIKOTE 862 having similar solubilities in water, dilutions of the saturated solution were prepared at 500, 250, 125, 31 mg/l for the D. magna and S. capricornutum tests, and in addition, 15.6 mg/l for the test with O. mykiss, in dilution water/culture medium. After mixing the contents of each vessel were allowed to stand for 1 hour before drawing-off the aqueous phase for subsequent testing.

2.2.3 Test methods

The range of concentrations used in the definitive tests that are described below were based upon the results of range-finding work.

O. mykiss: A sealed 96 h semi-static toxicity test was carried out with daily renewal of the test media. The test vessels were 11 litre full-volume, spherical, glass reaction vessels which were completely filled with test medium. Quantities of dilutions of the saturated solution of EPIKOTE 862 were added to the test vessels to give an approximately geometrically spaced series ranging from 500 to 15.6 mg/l. An additional vessel received no EPIKOTE 862 and served as a control. Ten O. mykiss were placed in each vessel. The vessels were then sealed with glass plates ensuring that all air was excluded. The fish were not fed during the test.

At points throughout the test (3, 24, 48, 72 and 96 h) the fish were observed and the numbers exhibiting toxic symptoms were recorded. The symptoms were classified into one of five categories from (a) - no toxic symptoms to (e) - dead (See Table 1). At 24 h intervals any dead fish were removed and surviving fish were transferred to freshly prepared media. The dissolved oxygen concentration and pH of the media in each test vessel were determined at the start and end of the test and prior and subsequent to each renewal of the test media. The total hardness and residual chlorine concentration were determined in all fresh test media. The temperature of water in an aquarium adjacent to the test vessels was determined at hourly intervals throughout the test.

D. magna: A sealed 48 h static toxicity test was carried out without renewal of the test media. The test vessels were 150 ml Erlenmeyer flasks which were completely filled with test medium. Quantities of the saturated solution of EPIKOTE 852 and dilutions of it were added to the flasks to give approximately geometrically spaced series of application rates ranging from 1000 to 31 mg/l. There were two replicate flasks at each test concentration. Two further flasks received no EPIKOTE 862 and served as controls. Ten D. magna, less than 24 h old were added to each flask. The flasks were then sealed with black screw-on lids that excluded air from the vessel. After 24 and 48 h the numbers of immobilised D. magna were recorded. D. magna were considered to be immobile if, when the contents of the flask were briefly swirled they did not swim during a 15 second period of observation. The pH and concentration of dissolved oxygen were determined in each of the test media at the start and end of the test. The total hardness of the control medium was determined at the start of the test. The temperature of water in a flask adjacent to the test vessels was determined at hourly intervals throughout the test.

S. capricornutum: A 3 day sealed growth inhibition test was carried out. The test vessels were 300 ml full-volume Erlenmeyer flasks which were completely filled with test medium. Quantities of the saturated solution of EPIKOTE 862 and dilutions thereof in algal growth medium were added to the flasks to give approximately geometrically spaced series of application rates ranging from 1000 to 31 mg/l. There were four flasks at each concentration. Seven further flasks received no EPIKOTE 862 and served as controls. Three out of each set of four flasks containing EPIKOTE 862 and

six of the control flasks were then inoculated with sufficient S. capricornutum cells to achieve a concentration of 5000 cells/ml. The cell concentration in each flask was then checked using a Coulter Multisizer (Appendix B). The remaining flasks (one control and six flasks containing EPIKOTE 862) were not inoculated and were used to determine particle counts in the absence of S. capricornutum. Two glass marbles were placed in each flask to ensure good mixing during incubation. The flasks were sealed with glass stoppers and then incubated in a cooled orbital incubator (100 cycles/min) under constant illumination (~3000 lux) at a nominal temperature of 22-26°C.

At the end of the test the growth of the cultures in the flasks was determined by measuring the chlorophyll a concentration of a sample taken from each flask. Chlorophyll a concentration was determined by boiling the sample with methanol, filtering it and measuring the absorbance of the filtrate with light of wavelength 665 nm and 750 nm (Talling and Driver 1963).

At the start and finish of the test the pH of each test medium was determined using the method described in Appendix C. The temperature in the test incubator was monitored at hourly intervals throughout the test.

2.3. Chemical analysis

For each of the toxicity tests samples of the test and control media were analysed at the start and conclusion of each exposure period to determine the concentration of EPIKOTE 862 present. Full details of the sampling and analytical method procedures are presented in Appendix D.

2.4 Statistics

O. mykiss: The 24, 48, 72 and 96 h LC_{50} values (those concentrations causing a 50% mortality after 24, 48, 72 and 96 h exposure) were calculated with their 95% confidence limits using the moving average angle method (U.S. Environmental Protection Agency, 1985).

D. magna: The 24 and 48 h LC_{50} values (those concentrations causing immobilisation of 50% of the D. magna exposed for 24 and 48 h) were calculated with their 95% confidence limits using the moving average angle method (U.S. Environmental Protection Agency, 1985).

S. capricornutum: Effects were evaluated by expressing chlorophyll a concentration as a percentage of mean control chlorophyll a concentration. 72 h EC_{50} values (those concentrations causing a 50% reduction in growth by comparison with mean control growth after 72 h) were calculated with their 95% confidence limits by probit analysis using log_{10}. transformed concentration values (Finney, 1971). The highest no observed effect concentration was calculated using Williams' test (Williams, 1971, 1972).

3. RESULTS

3.1 Water quality

The results of the water quality determinations are summarised below and presented in full in Appendix C.

Parameter		O. mykiss	D. magna	S. capricornutum
Temperature (°C)		16 to 17	21 to 23	21 to 24
pH		6.9 to 7.9	7.7 to 8.5	8.0 to 10.3
Dissolved oxygen	(mg/l)	7.9 to 10.4	8.0 to 9.2	N/D
Total hardness (as $CaCO_3$)	(mg/l)	254 to 268	174	N/D
Residual chlorine	(mg/l)	0.02 to 0.04	N/D	N/D

(N/D not determined)

The water quality parameters were generally in the preferred ranges for all of the tests. However the following should be noted.

The total water hardness recorded during the test with O. mykiss was above the preferred maximum of 250 mg/l ($CaCO_3$) according to the OECD guideline 203 (Fish, Acute Toxicity Test). The recorded range of 254 to 268 was not regarded sufficient to jeopardise the outcome of the study. The pH recorded during the test with S. capricornutum was within the OECD guideline 207 (Alga, Growth Inhibition Test) of ± 1 pH unit between all flasks at the start of the test. By the end of the test, 72 h later, the pH in the flasks containing the control medium and at measured concentrations of 0.24, 0.48, 0.93 and 2.0 mg/l EPIKOTE 862 had risen by more than 1 unit. This reflects good algal growth and is not regarded as sufficient to jeopardise the outcome of the study.

3.2 Toxicity tests

3.2.1 Analysis of aquatic toxicity test media

The results of the analysis of the aquatic toxicity test media are presented in Appendix D.

Figure D1, shows HPLC chromatograms of a standard solution of EPIKOTE 862 and of extracts from fresh and aged saturated solutions. The ageing of solutions appeared to provide shorter retention time, probably more water soluble material, probably alcohols formed by hydrolytic opening of the opoxide groups. These were not present to any significant extent in the EPIKOTE 862 standard. Because they were produced from the major components of the test substance, the concentration of these shorter retention time components would be a function of the amounts of the major components of EPIKOTE 862 which dissolved, and not of the loading rate used to prepare the water accommodation fraction. For that reason, dilution of a saturated solution was the appropriate experimental technique to use for this study.

For the test with O. mykiss, dilution of the saturated solution of EPIKOTE 862 prepared at 1000 mg/l resulted in a series of mean measured concentrations ranging from 4.4 to 0.1 mg/l at the start of the 24 h exposure periods. At the start of the first exposure period, the mean measured concentrations were about 50% of the mean measured concentrations at the start of the subsequent three exposure periods. The reasons for this were not investigated further. By the end of each exposure period measured concentrations fell by an average of 11 to 25% of the initial measured concentration.

For the test with D. magna, the saturated solution of EPIKOTE 862, prepared at 1000 mg/l, and the dilutions, resulted in a series of measured concentrations ranging from 7.4 to 0.24 mg/l at the start of the 48 h exposure period. The fall in concentrations over the 48 h exposure period ranged from 8 to 26% of the initial measured values.

For the test with S. capricornutum, the saturated solution of EPIKOTE 862, prepared at 1000 mg/l, and the dilutions, resulted in a series of measured concentrations ranging from 10 to 0.31 mg/l at the start of the 72 h exposure period. The fall in concentrations over the 72 h exposure period ranged from 34% to 44% of the initial measured value.

The results of the toxicity tests are expressed in relation to measured concentrations of EPIKOTE 862 in the test media.

3.2.2 Oncorhynchus mykiss

The results of the toxicity test with O. mykiss are given in Table 1. Figure 1 is a plot of mortality of O. mykiss after 96 h exposure to a range of measured concentrations of EPIKOTE 862.

All of the O. mykiss died after 4 h exposure to 4.0 mg/l of EPIKOTE 862. By the end of the test all of the O. mykiss had died at 4.0, 1.2 and 0.97 mg/l and 50% had died at 0.55 mg/l. There were no mortalities or sublethal effects of EPIKOTE 862 to O. mykiss at 0.24 and 0.16 mg/l.

The LC_{50} values, calculated on the basis of mean determined concentrations of EPIKOTE 962 in the test media together with their 95% confidence intervals are summarised below

	LC_{50} (mg/l)	95% Confidence interval (mg/l)
24 h	1.1	0.99-1.28
48 h	0.73	0.58-0.93
72 h	0.55	0.41-0.69
96 h	0.55	0.41-0.69

3.2.3 Daphnia magna

The results of the toxicity test with D. magna are given in Table 2. Figure 2 is a plot of the percentage of D. magna immobilised after 48 h exposure to a range of mean measured concentrations of EPIKOTE 862.

After 48 h exposure to EPIKOTE 862 all of the D. magna were immobilised at 6.6 and 3.4 mg/l. At 1.6 mg/l of EPIKOTE 862, 55% of the D. magna were immobilised by the end of the 48 h exposure period.

The 24 and 48 h EC_{50} values, calculated on the basis of mean determined concentrations of EPIKOTE 862 in the test media over the duration of the test were 3.2 mg/l and 1.6 mg/l respectively (95% confidence intervals 2.4 to 4.7 mg/l and 1.2 to 2.2 mg/l respectively).

3.2.4 Selenastrum capricornutum

The results of the toxicity test with S. capricornutum are given in Table 3. Figure 3 is a plot of the measured concentration of EPIKOTE 862 versus growth inhibition measured by the concentration of chlorophyll a in the test cultures at the end of the test. Based on the reduction in chlorophyll a relative to controls EPIKOTE 862 in the test media, the 72 h EC_{50} to S. capricornutum was calculated to be 1.8 mg/l (95% confidence intervals 1.5 to 2.1 mg/l).

The highest no observed effect concentration (NOEC) of EPIKOTE 862 to S. capricornutum was calculated to be 0.24 mg/l.

Signed: _____ (Study Director)

Date: 1/07/93.

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Table 1 - Toxic symptoms exhibited by O. mykiss exposed to a range of concentrations of EPIKOTE 862

Mean measured concentration of EPIKOTE 862 (mg/l)	Number of fish	Observation time and symptom classification*																								
		3 h					24 h					48 h					72 h					96 h				
		a	b	c	d	e	a	b	c	d	e	a	b	c	d	e	a	b	c	d	e	a	b	c	d	e
Control	10	10					10					10					10					10				
0.16	10	10					10					10					10					10				
0.24	10	10					10					10					10					10				
0.55	10	10					10					10					5			5		5				5
0.97	10	10						9	1								10				10					10
1.2	10	10									10						10				10					10
4.0	10			6	4						10						10				10					10

* Symptoms classification

(a) Number of fish exhibiting no toxic symptoms.
(b) Number of fish swimming normally but exhibiting toxic symptoms e.g. increased cough frequency, hyperventilation.
(c) Number of fish swimming abnormally e.g. on side or back.
(d) Number of fish immobilised e.g. lying on bottom of tank or floating at surface, but still alive. Fish in this category were removed from the vessels and their numbers added to the total in category 'e' at subsequent observations.
(e) Number of fish dead.

Table 2 - Immobilisation of D. magna exposed to a range of concentrations of EPIKOTE 862

Mean measured concentration of EPIKOTE 862 (mg/l)	Number of D. magna	Number immobilised	
		24 h	48 h
Control	10	0	0
	10	0	0
0.23	10	0	0
	10	0	0
0.4	10	0	0
	10	0	0
0.79	10	0	0
	10	0	0
1.6	10	0	5
	10	0	6
3.4	10	9	10
	10	3	10
6.6	10	10	10

Table 3 - Growth of S. capricornutum cultures exposed to a range of concentrations of EPIKOTE 862

Mean measured concentration of EPIKOTE 862 (mg/l)	Chlorophyll a concentration at t = 72 h (µg/l)	Mean chlorophyll a concentration (µg/l)	Chlorophyll a concentration as a % of controls	Mean percent reduction in chlorophyll a compared to controls
Control	130 139	128	-	-
	146 121			
	107 125			
0.24	126	115	99	10
	103		81	
	116		91	
0.48	121	103	95	19
	115		90	
	74		58	
0.93	110	99	86	22
	98		66	
	89		70	
2.0	*	61	-	52
	48		37	
	74		58	
4.2	35	35	28	72
	31		25	
	39		31	
6.3	21	13	17	89
	9		7	
	10		8	

* Sample mistakenly discarded.

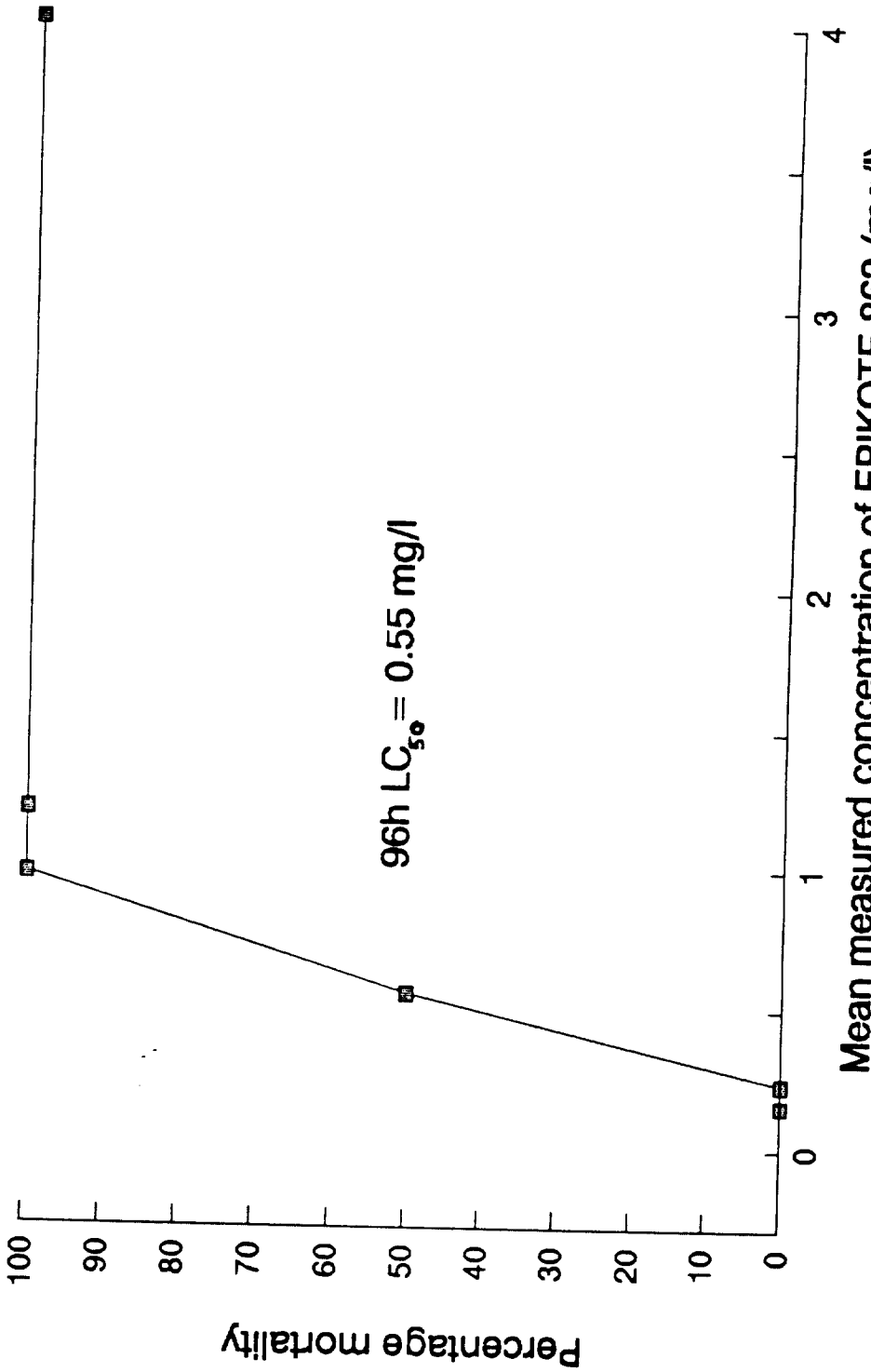


Figure 1. Percentage mortality of O. mykiss after 96h exposure to a range of concentrations of EPIKOTE 862.

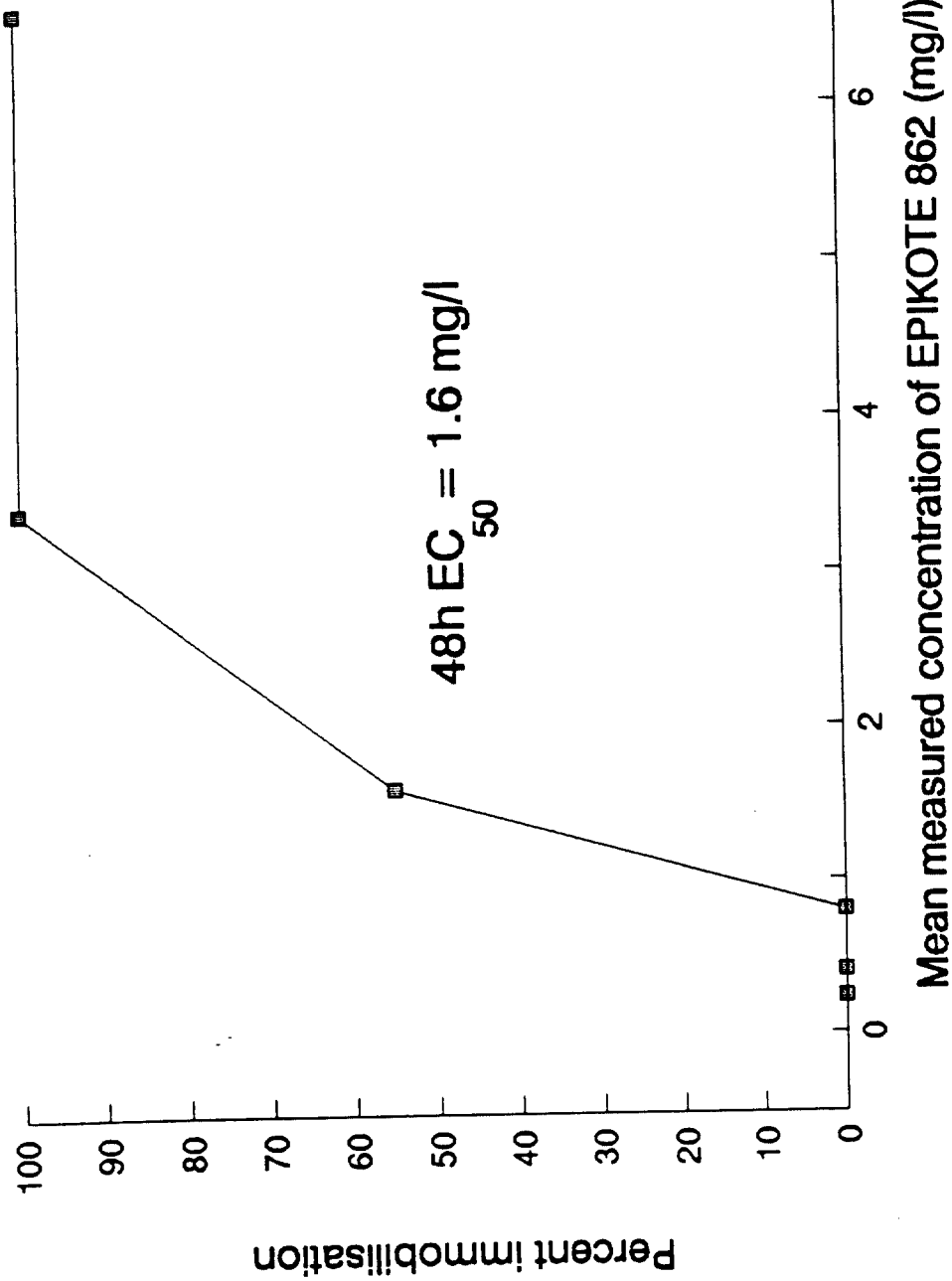


Figure 2. Percentage immobilisation of D. magna after 48h exposure to a range of concentrations of EPIKOTE 862.

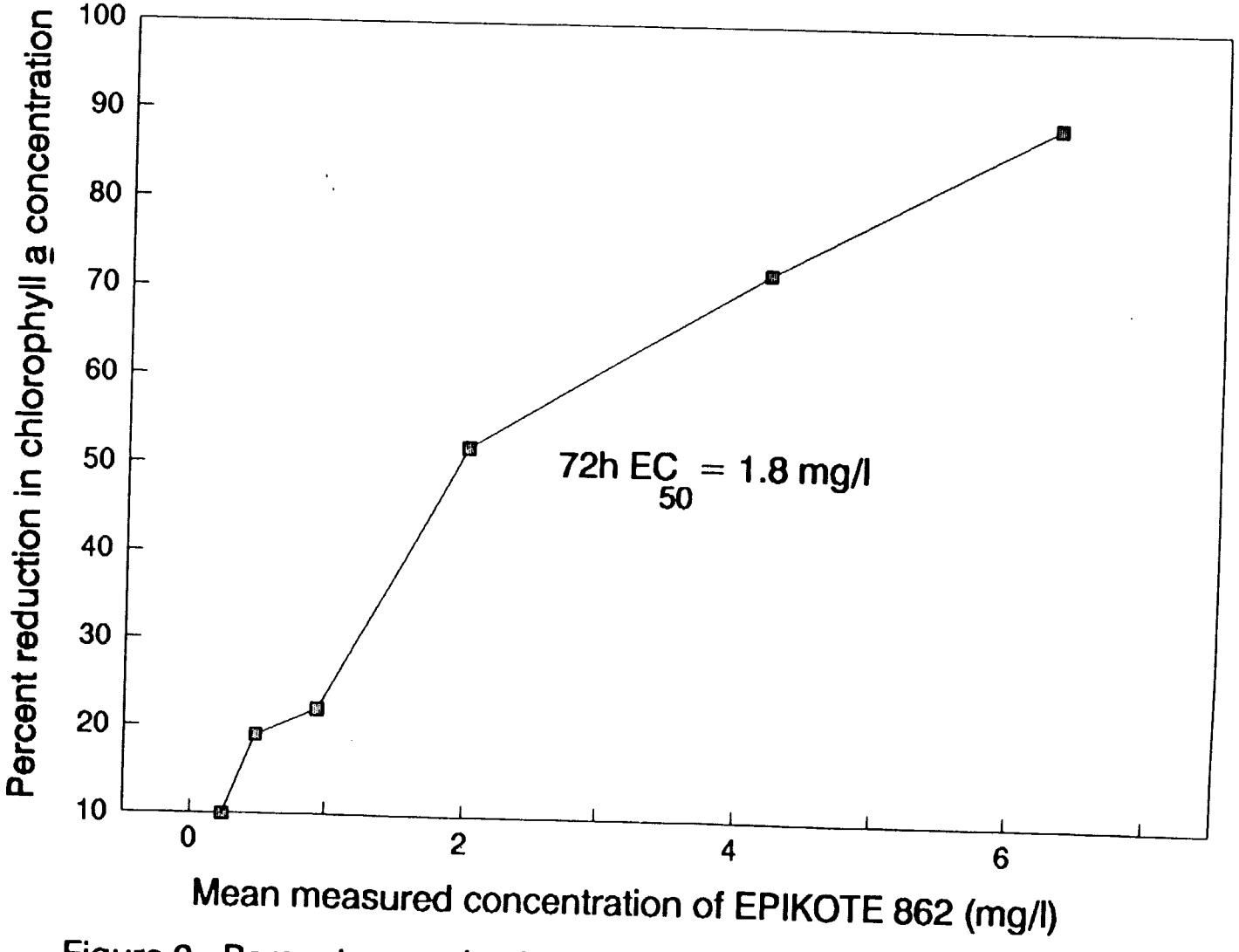


Figure 3. Percentage reduction in chlorophyll a of S.capricornutum relative to controls after 72h exposure to a range of concentrations of EPIKOTE 862.

APPENDIX A

Title: COMPOUND CONTROL REPORT (Expt. Number 4607)

Test substance

Identity of the test substance

The data for the test substance released for use in this experiment are tabulated below.

NAME	EPIKOTE 862
CODE NUMBER	L1262
BATCH (& OTHER) NUMBERS	Tank 1126; Indent 9450/9909
TOXICOLOGY REF. NUMBER	ST92/100
SOURCE	Shell Nederland Chemie B.V., Pernis
DATE RECEIVED	31st March 1992
APPEARANCE	Clear colourless viscous liquid
CHARACTERIZATION	**Actual analysis**

Viscosity @ 25°C	3.51 Pa.s	(ASTM D 445)
Epoxy group content	5900 mmol/kg	(SMS 2026)
Colour (Pt/co)	45	(ISO 2211)

Ref. Certificate of analysis dated 24-03-92 supplied by Shell Nederland Chemie B.V.

No claim of GLP compliance is made in respect of these data.

DATE RELEASED	9th April 1992

Storage of this test substance

Following its arrival in Compound Control this test substance was stored in the dark at ambient temperature.

Stability of this test substance

The data sheet (EK1.1.102) supplied with this sample stated that, provided EPIKOTE 862 was stored at normal temperatures in such a way that moisture is excluded, the storage life should be at least one year. The storage conditions employed complied with this provision, and therefore I consider that the test substance was stable for the duration of this study.

Responsible Practitioner

(signature)

30 October 1992

(date)

APPENDIX B

Title: MAINTENANCE/CULTURE OF TEST ORGANISMS (Expt. Number 4607)

The test organisms used in this experiment were maintained and cultured as described below.

1. Procedure for the maintenance of Oncorhynchus mykiss Walbaum

O. mykiss for use in toxicity tests are obtained from commercial hatcheries. On arrival at the laboratory the fish are given a batch number and are inspected for signs of disease. The fish are placed in ~300 l, circular, self-cleaning, fibre-glass tanks at a density appropriate to their size. The tanks receive a continuous flow of temperature controlled water of a suitable and defined quality (Appendix C). The fish are fed a maintenance ration of Mainstream Trout Food No. 03 (BP Nutrition Ltd.), the quantities fed depending upon the size and age of the fish and the temperature of the water. Fish mortalities and dissolved oxygen concentration in the tanks are recorded daily. The fish are held in a temperature controlled room, 13-17°C, under artificial light with a 16 h light 8 h dark cycle. The fish are deemed acceptable for testing if the cumulative mortality in the batch is less than 5% over a 7 day period preceding the test. The stock is not used for testing if mortality exceeds 5% or if disease is apparent.

After a 48 h settling-in period, the fish are acclimated to the test conditions for a minimum of 7 days before a test begins. They are not fed in the 24 h preceding a test. At the conclusion of each test a sample of ten fish used in the test are weighed (wet weight) and measured (fork length).

2. Procedure for the culture of Daphnia magna (Straus)

The laboratory culture of D. magna is derived from a clone held at ICI Brixham Laboratory. The ICI clone was itself obtained from the Institut National de Recherche Chimique Applique (I.R.Ch.A.), France.

For the first 14 days the D. magna are cultured in 1 litre pyrex glass beakers containing 0.8 litre of reconstituted freshwater (Appendix C). From Day 15 onwards they are cultured in a 2 litre beaker containing 1.5 litre of reconstituted freshwater. Each vessel and its contents are referred to as a 'culture'. New cultures are started with animals less than 24 h old, at a density of about 12 per litre. The cultures are held in a temperature controlled room, nominally 18-22°C, under artificial light in a 16 h light 8 h dark cycle. The cultures are fed daily with a concentrated suspension of Chlorella vulgaris to give a concentration of approximately 0.10×10^6 cells/ml. The C. vulgaris is obtained from a 5 litre culture grown under semi-axenic conditions. Prior to use the C. vulgaris is concentrated to about $100\text{-}200 \times 10^6$ cells/ml by filtration and resuspension in reconstituted freshwater (Appendix C). The C. vulgaris is stored in a refrigerator at 4°C and used within one week.

The water in the culture vessels is renewed twice weekly. Young are removed daily using a pipette (if not required for testing) or sieve (56 mesh/cm). Cultures are discarded when 28 days old.

Young for use in acute toxicity tests are collected from the third brood onwards of cultures aged between 15 and 28 days. 24 h before a test is set up, any young present in the cultures are removed and discarded. Approximately 23 h later, young for use in the test are removed from the parent culture vessel and transferred to fresh culture medium. The young are then left for one hour before selecting actively swimming individuals for use in the test.

Young for testing are not taken from cultures which contain adults bearing ephippia; these cultures are discarded.

3. Procedure for the culture of Selenastrum capricornutum (Prinz)

Axenic stock cultures of S. capricornutum are maintained on agar plates. These are used to inoculate liquid cultures which, while in exponential phase growth, are in turn used to inoculate test solutions.

Source

The axenic strain of S. capricornutum (ATCC 22662) was obtained from the American Type Culture Collection, Maryland, Ohio, U.S.A.

Maintenance of cultures

Agar plates are prepared by adding 1.5% mass/volume agar to liquid medium (Appendix C) prior to autoclaving. On cooling the agarified medium is poured into 9 cm diameter sterile plastic petri dishes and allowed to set. The algal cultures are streaked onto these plates and maintained under continuous illumination at 18-22°C in a Gallenkamp vertical incubator. The cultures are renewed approximately every fortnight.

Cultures in liquid medium (Appendix C) are initiated with cells transferred on a sterile loop from an agar plate. The cultures are grown as 100 ml batch cultures in 250 ml Erlenmeyer flasks for up to 4 days. The cultures are maintained at 22-26°C in a Gallenkamp orbital incubator under constant illumination (~ 3000 lux).

Inoculation of test cultures

The quantity of inoculum introduced into each test vessel is sufficient to give a concentration of 5000 cells ml/l. The cell concentration of an exponentially growing stock culture is measured using a Coulter Multisizer and the required inoculum volume calculated. All flasks used for a particular test are inoculated from the same stock culture.

APPENDIX C

Title: WATER QUALITY (Expt. Number 4607)

INTRODUCTION

The quality of the medium in which organisms are held, prior to and during toxicity tests, can influence the results of the tests. This report gives details of the quality of the media used for acclimation/culture, and for testing, in the experiments described in the main report.

MATERIALS AND METHODS

Source and treatment

Oncorhynchus mykiss

The water used for maintenance of stocks and for the toxicity test with O. mykiss is the laboratory mains supply. The water comes from two pumping stations (Highstead and Eastling) controlled by the Mid Kent Water Company. The water is obtained from bore holes in the chalk of the North Downs. The only chemical treatment prior to its arrival in the laboratory is chlorination to 0.1 mg/l.

In the laboratory the water is filtered (PALL MDY 1001 Y400) to remove all particles larger than 15 μm (90% of particles greater than 10 μm) and passed through activated carbon filters (Cano model CT) to remove chlorine and organic contaminants. Both particle and activated carbon filter cartridges are renewed as recommended by the manufacturers. Heat exchange units (stainless steel and perspex) are used to adjust the temperature of the water. Prior to use in tests the water is vigorously aerated for several hours to ensure that no residual chlorine remains.

Samples of the water, after filtration but before aeration, are taken at approximately 6 month intervals and analysed for a number of parameters (Table C.1). These analyses are carried out by Mid Kent Scientific Services Limited.

Daphnia magna

The water used for the culturing and testing of D. magna is a reconstituted fresh water prepared by dissolving the following amounts of Analar grade salts in Millipore "Milli-Q" filtered water:-

$NaHCO_3$	192 mg/l
$CaSO_4.2H_2O$	120 mg/l
$MgSO_4.7H_2O$	240 mg/l
KCl	8 mg/l

This recipe has been recommended as one suitable for producing a 'hard' water by the U.S. Environmental Protection Agency (1975).

Before use for culturing, a soil extract is added to the reconstituted fresh water at a rate of 20 ml/l. The soil extract is prepared by autoclaving 100 g soil per litre of Millipore "Milli-Q" filtered water for 15 minutes at 120°C. Solids are removed by filtration through Whatman GF/C paper.

Selenastrum capricornutum

A nutrient medium is prepared by dissolving Analar grade salts in Millipore "Milli-Q" filtered water. Nutrient concentrations are those described by Miller and Green (1978) except that boric acid is present at 105 µg/l (184 µg/l in Miller and Green), and sodium bicarbonate at 50 mg/l (15 mg/l in Miller and Green).

The medium (excluding sodium bicarbonate) is autoclaved at 1.0 kg/cm^2 for 15 min. On cooling, 20 ml/l of a 0.45 µm millipore-sterilised solution of sodium bicarbonate (2.5 g/l) is added to yield a final concentration of 50 mg/l in the nutrient medium.

Water quality during stock culture, maintenance and acclimation

O. mykiss: The temperature of the water is monitored at hourly intervals by a computer controlled thermocouple system which outputs to a dedicated microcomputer. Temperature data are stored on the hard disc and can be retrieved either as hard copy for visual inspection and reporting or on magnetic tape for archiving. The pH, concentration of dissolved oxygen, and total hardness are checked weekly. The pH measurements are made with a calibrated pH meter. The dissolved oxygen measurements are made with a YSI 57 dissolved oxygen meter calibrated in air saturated water immediately prior to use. Total hardness is determined by titration against EDTA in the presence of ammonia buffer and a suitable indicator.

D. magna: The temperature of water in a beaker adjacent to the cultures is monitored and the pH, dissolved oxygen concentration and total hardness of each batch of culture medium used are checked, using the system and procedures described for O. mykiss.

S. capricornutum: The temperature in the incubator is monitored using the system described for O. mykiss. The pH of the media is checked prior to use with a calibrated pH meter.

Water quality during tests

O. mykiss: The water temperature in a vessel adjacent to the test aquaria was monitored as described previously. The pH and dissolved oxygen concentration was determined in the control and each test media at the start and conclusion of each exposure period. The total hardness and residual chlorine concentrations were determined for each new batch of dilution water used. Residual chlorine concentration is determined using a BDH Lovibond Nesslerizer. All other determinations are made using the methods described previously.

D. magna: The temperature of water in a vessel adjacent to the test vessels was monitored as described previously. The pH and concentration of dissolved oxygen in the control and each test media were determined at the start and conclusion of the exposure period. The total hardness of the water used in the test was determined. All determinations were made using the methods described previously.

S. capricornutum: The temperature in the orbital incubator was monitored as described previously. The pH of the control and each test media was determined at the start and conclusion of the exposure period.

RESULTS

Quality of laboratory mains supply

The most recent data set and the range of values obtained since monitoring of the laboratory water supply began are given in Table C1.

Water quality during the acclimation of O. mykiss and the culture of D. magna and S. capricornutum

The results of the determinations are given in Table C2 (O. mykiss), Table C3 (D. magna) and Table C4 (S. capricornutum).

Water quality during toxicity tests

The results of the determinations are given in Table C5 (O. mykiss), Table C6 (D. magna) and Table C7 (S. capricornutum).

REFERENCES

Miller, W. E. and Green, J. C. (1978).
The Selenastrum capricornutum (Prinz) algal bottle test.
EPA-600/9-78-018.

U.S. Environmental Protection Agency (1975).
Methods for acute toxicity testing with fish, macro-invertebrates and amphibians.
EPA-660/3-75-009.

Table C1 - Quality of laboratory mains supply, after filtration (10 μm) and passage through activated carbon.

Parameter	Overall range (12/10/89 - 26/5/92) (n = 5)	Latest value (26/5/92)	
CONDUCTIVITY	519 - 534	522	(μs/cm)
pH	7.0 - 7.5	7.4	
SAT. INDEX	-1.08 - -0.1	ND	(NO)
TOTAL SOLIDS	205 - 365	364	(mg/l)
ALKALINITY	254 - 264	264	(mg/l)
CARBON DIOXIDE	ND - ND	ND	(mg/l)
TOTAL HARDNESS	273 - 285	285	(mg/l)
PERMANGANATE VALUE	<0.04 - 0.12	ND	(mg/l)
TOTAL CHLORINE	<0.02 - 0.02	<0.02	(mg/l)
PHENOL	<0.5 - <5	<5	(μg/l)
TOTAL CALCIUM	106 - 110	110	(mg/l)
TOTAL MAGNESIUM	1.6 - 2.6	2.6	(mg/l)
TOTAL IRON	<0.01 - <0.02	<0.01	(mg/l)
TOTAL MANGANESE	<0.02 - <0.02	<0.02	(mg/l)
TOTAL MERCURY	<0.2 - <0.2	<0.2	(μg/l)
TOTAL LEAD	<5 - <5	<5	(μg/l)
TOTAL CADMIUM	<0.5 - <0.5	<0.5	(μg/l)
TOTAL ARSENIC	<5 - <5	<5	(μg/l)
TOTAL COPPER	<0.02 - 0.09	<0.02	(mg/l)
TOTAL ZINC	<0.02 - 0.03	0.03	(mg/l)
TOTAL SODIUM	10 - 11	10	(mg/l)
TOTAL POTASSIUM	1.0 - 1.4	1.1	(mg/l)
CHLORIDE	17 - 19	19	(mg/l)
FLUORIDE	0.09 - 0.10	0.09	(mg/l)
NITRATE	3.9 - 4.0	4.0	(mg/l)
NITRITE	<1 - 4	<1	(μg/l)
ORTHO PHOSPHATE	0.03 - 0.04	0.04	(mg/l)
TOTAL SILICA	9.6 - 11.4	9.6	(mg/l)
SULPHATE	4 - 6	6	(mg/l)
AMMONIACAL N	<0.002 - <0.01	<0.01	(mg/l)
ALBUMINOID N	<0.002 - <0.002	ND	(mg/l)

ND = not determined

Table C2 - Water quality during the acclimation of the O. mykiss

i.e. two most recent sets of readings prior to test

Date	24.8.92	2.9.92
Temperature (°C) [monitored at hourly intervals]	15 - 17	
Total water hardness (mg/l as $CaCO_3$)	272	178
pH	7.8	7.3
Concentration of dissolved oxygen (mg/l)	9.8	9.8

Table C3 - Water quality during the culture of the parents of

the D. magna used in the test

Date	23.6.92	26.6.92
Temperature (°C) [monitored hourly intervals]	19 - 20	
Total water hardness (mg/l as $CaCO_3$)	170 - 176	
pH	8.1 - 8.1	
Concentration of dissolved oxygen (mg/l)	10.4 - 10.6	

Table C4 - Media quality during the growth of the

S. capricornutum starter culture

Nominal temperature range (°C)	21 - 23
pH prior to use	9.7

Table C5 - Water quality during the test with O. mykiss

Time (h)		0	24	48	72	96
Solution tested		Fresh	Old/Fresh	Old/Fresh	Old/Fresh	Old
Temperature (°C) [Monitored at hourly intervals]		16 - 17				
Total hardness (mg/l as $CaCO_3$)		254	- /260	- / 268	- / 256	-
pH	Control	6.9	7.7/7.3	7.6/ 7.4	7.6/ 7.5	7.7
	31.2 mg/l	7.4	7.6/7.3	7.3/ 7.5	7.4/ 7.6	7.8
	62.5 mg/l	7.4	7.2/7.4	7.4/ 7.5	7.7/ 7.5	7.9
	125 mg/l	7.3	7.7/7.3	7.6/ 7.5	7.8/ 7.5	7.9
	250 mg/l	7.3	7.6/7.4	7.7/ -	- / -	-
	500 mg/l	7.3	7.7/ -	- / -	- / -	-
	1000 mg/l	7.3	7.8/ -	- / -	- / -	-
Conc. of dissolved oxygen (mg/l)	Control	10.4	8.9/10.2	8.7/10.0	8.6/ 9.8	8.7
	31.2 mg/l	10.2	8.7/10.2	8.5/ 9.8	8.3/ 9.9	8.3
	62.5 mg/	10.2	8.4/ 9.9	8.3/ 9.7	8.4/ 9.9	7.9
	125 mg/l	10.4	8.2/10.2	8.3/ 9.9	8.7/ 9.9	8.2
	250 mg/l	10.2	8.0/10.4	8.2/ -	- / -	-
	500 mg/l	9.9	8.9/ -	- / -	- / -	-
	1000 mg/l	9.8	9.2/ -	- / -	- / -	-
Residual chlorine conc. (mg/l)		0.04	- /0.04	- / 0.04	- / 0.02	-

Table C6 - Water quality during the test with D. magna

Time (h)		0	48
Temperature (°C) [Monitored at hourly intervals]		21 - 23	
Total hardness (mg/l as $CaCO_3$)		174	
pH	Control	8.2	8.4
	0.23 mg/l	7.7	8.5
	0.4 mg/l	7.8	8.5
	0.79 mg/l	8.1	8.5
	1.6 mg/l	8.2	8.5
	3.4 mg/l	8.2	8.5
	6.6 mg/l	8.3	8.5
Conc. of dissolved oxygen (mg/l)	Control	9.2	9.0
	0.23 mg/l	8.7	8.2
	0.4 mg/l	8.4	8.0
	0.79 mg/l	8.7	8.3
	1.6 mg/l	8.8	8.4
	3.4 mg/l	8.6	8.2
	6.6 mg/l	8.4	8.0

Table C7 - Temperature and pH during the test with S. capricornutum

Time (h)		0	72
Temperature(°C)[Monitored at hourly intervals]		21 - 24	
pH	Control	8.0	10.1
	0.24 mg/l	8.6	10.1
	0.48 mg/l	8.6	10.3
	0.93 mg/l	8.3	10.2
	2.0 mg/l	8.3	9.8
	4.2 mg/l	8.3	9.2
	6.3 mg/l	8.3	8.7

Title of Main Report: EPIKOTE 862: acute toxicity to Oncorhynchus mykiss, Daphnia magna and Selenastrum capricornutum

Experiment Number: 4607

APPENDIX D

Author: D.D. Lad

Scientific Reviewer: M.K. Baldwin

Title of Appendix: Chemical analysis of test media

Summary: An HPLC procedure for the analysis of the test media for Epikote 862 is described, and the results of the analyses are reported.

[redacted]

(Signature) (Date)

1. INTRODUCTION

A high-performance liquid chromatographic (HPLC) method was developed to determine the concentration of EPIKOTE 862 in water. The substance is extracted from the water using a Whatman ODS-2 octadecyl solid phase extraction cartridge, then analysed by HPLC using a reversed phase column with ultraviolet absorption detection. Calibration is by external standardisation with solutions of EPIKOTE 862. The method is shown as Attachment D1. The results of application of this method to the analysis of aqueous test media from toxicity tests with Oncorhynchus mykiss, Daphnia magna and Selenastrum capricornutum are reported. The test media were saturated solutions of EPIKOTE 862 or dilutions thereof. The highest concentration test medium was produced by mixing 1000 mg of the test substance per litre of untreated medium, then using the aqueous phase. The lower concentrations were produced by diluting the highest concentration medium with untreated medium.

2. EXPERIMENTAL

a. Sampling and analysis programme for the test media

O. mykiss

The test media samples from the O. mykiss tests were prepared freshly each day for the duration of the test, which began on 7th September and finished on 11th September 1992. On each occasion a single sample of about 250 ml was taken from the fresh and the old media with application rates of 0, 31.25, 62.5, 125, 250, and 500 mg/l, except where certain application rates had been discontinued.

The test media samples from the D. magna and S. capricornutum tests were taken at the start and at the end of the exposure periods. Samples for analysis from these tests were received as follows:-

D. magna

30th June 1992, start of study (T = 0), one sample each from application rates of 0, 31.25, 62.5, 125, 250, 500, and 1000 mg/l.

2nd July 1992, end of study, (T = 48 hours), one sample each from application rates of 0, 31.25, 62.5, 125, 250, 500, and 1000 mg/l.

S. capricornutum

7th July 1992, start of study (T = 0), one sample each from application rates of 0, 31.25, 62.5, 125, 250, 500, and 1000 mg/l.

10th July 1992, end of study (T = 72 hours), one sample each from application rates of 0, 31.25, 62.5, 125, 250, 500, and 1000 mg/l.

Note:- A portion of the control medium taken at each sampling time was used for the determination of the percentage recovery during the analysis procedure.

All samples were analysed on the day of receipt.

b. Analysis recovery determinations performed with test media analyses

Analysis recovery determinations were performed by adding, at the time of analysis, a known amount of the test substance in acetonitrile to a sample of control medium.

3. RESULTS

a. Results of analysis of samples from the aquatic toxicity studies

The HPLC chromatogram of EPIKOTE 862 is complex, with at least nine components being visible. Four of them are large compared with the rest, and the concentrations of EPIKOTE 862 in samples are measured using those four peaks. It is assumed that the extinction coefficients for UV light at 230 nm are the same for equal weights of the four major components. That is a reasonable assumption for an approximation, since the absorptions are caused, predominantly, by the aromatic rings in the substance, and they will not be influenced much by isomerism. The larger molecular weight components will have more aromatic rings in them, because they contain more diphenylol methane units, so the absorption will rise as the molecular weight increases. A series of typical chromatograms is shown as Figure D1. Chromatograms from saturated solutions, especially aged ones, showed early peaks which were absent or at low abundance in the standard solutions. These were not included in quantitative measurements. The results of analysis of the test media for EPIKOTE 862 are shown in Tables D1, D2, and D3. They have been rounded up to two significant figures. Analytical recovery data are shown in Table D4, for which the addition concentrations were from 0.2 to 2.0 mg/litre. The percentage recoveries ranged from 109% to 90%.

For the test with O. mykiss, dilution of the saturated solution of EPIKOTE 862 prepared at 1000 mg/litre resulted in a series of mean measured concentrations ranging from 4.4 to 0.1 mg/litre at the start of each of the 24 h exposure periods.

At the start of the first exposure period, the mean measured concentrations of the dilutions, which ranged from 125 to 15.6 mg/litre, were about 50% of the mean measured concentrations at the start of the subsequent three exposure periods. The reasons for this were not investigated further.

By the end of each exposure period, measured concentrations fell by an average of 11% to 25% of the initial measured concentration.

For the test with D. magna, the saturated solution of EPIKOTE 862, prepared at 1000 mg/litre and then diluted, resulted in a series of measured concentrations ranging from 7.4 to 0.24 mg/litre at the start of the 48 h exposure period. The fall in concentration over the 48 h exposure period ranged from 8% to 26% of the initial measured value.

For the test with S. capricornutum, the saturated solution of EPIKOTE 862, prepared at 1000 mg/litre and then diluted, resulted in a series of measured concentrations ranging from 10 to 0.31 mg/litre at the start of the 72 h exposure period. The fall in concentration over the 72 h exposure period ranged from 34% to 44% of the initial measured value.

Table D5 illustrates the way that HPLC profiles of extracts from fresh and old saturated solutions of Epikote 862 compare with those from standard solutions of that substance. The four components measured elute from the HPLC system in the order A, B, C, D and, together, they comprise about 88% of the total peak area. Comparing the relative areas of the four peaks for the standard solutions with those from the extracts obtained during the D. magna test media, which was chosen as being representative of all of the test media used in this study, there was little change in peak A, the size of peak B had increased by about 10%, and peak C had increased by about 30%. The relative size of peak D had approximately halved. The components eluting later than peak D, which were not quantified, were much less prominent in the extracts from the samples than in the standard solutions. These differences are probably a reflection of the different water affinities of the various components of Epikote 862. There were no major differences between the profiles obtained with extracts from fresh and old saturated solutions.

Table D1 - Results of analysis of test media for EPIKOTE 862 during the O. mykiss test

Nominal concentrations of EPIKOTE 862 (mg/l)	Concentration of EPIKOTE 862 (mg/l)								Geometric mean concentration				
	Day 0	Day 1		Day 2		Day 3		Day 4					
	Fresh	Old	Fresh	Old	Fresh	Old	Fresh	Old	Day 1	Day 2	Day 3	Day 4	Overall
0 (control)	<0.01	0.03	0.01	<0.01	<0.01	<0.01	<0.01	<0.01	-	<0.01	<0.01	<0.01	(<0.01)
15.6	0.10	0.12	0.22	0.20	0.16	0.16	0.19	0.16	0.11	0.21	0.16	0.17	0.16
31.25	0.16	0.15	0.32	0.22	0.31	0.21	0.36	0.26	0.16	0.27	0.26	0.30	0.24
62.5	0.39	0.27	0.66	0.47	0.73	0.78	0.76	0.53	0.32	0.56	0.75	0.65	0.55
125.0	0.66	0.45	1.91	1.60	-	-	-	-	0.54	1.7	-	-	0.97
250.0	1.27	1.05	-	-	-	-	-	-	1.2	-	-	-	1.2
500	4.45	3.50	-	-	-	-	-	-	4.0	-	-	-	4.0

Table D2 - Results of analysis of test media for EPIKOTE 862 during the D. magna test

Nominal concentrations of EPIKOTE 862 (mg/l)	Concentration of EPIKOTE 862 (mg/l)		Geometric mean mg/l
	Fresh medium (T=0)	End of test (T=2 days)	
0 (control)	<0.01	<0.01	<0.01
31.25	0.24	0.22	0.23
62.5	0.46	0.35	0.40
125.0	0.92	0.68	0.79
250.0	1.7	1.5	1.6
500.0	3.7	3.1	3.4
1000.0	7.4	5.9	6.6

Table D3 - Results of analysis of test media for EPIKOTE 862 during the S. capricornutum test

Nominal concentration of EPIKOTE 862 (mg/l)	Concentration of EPIKOTE 862 (mg/l) Fresh medium (T=0)	End of test (T=3 days)	Geometric mean
0 (control)	<0.01	<0.01	<0.01
31.25	0.31	0.19	0.24
62.5	0.64	0.36	0.48
125.0	1.2	0.71	0.93
250.0	2.5	1.6	2.0
500.0	5.4	3.3	4.2
1000	10	6.6	6.3

Table D4 - Results of recovery determinations with EPIKOTE 862 added to blank media

Test	Nominal concentration (mg/litre)	Recovery (percent of that added) Day 0	Day 1	Day 2	Day 3	Day 4
O. mykiss	0.2	109			104	93
O. mykiss	0.2	90			98	95
O. mykiss	2.0		93			
O. mykiss	2.0		92			
O. mykiss	2.0		95			
O. mykiss	0.5			107		
O. mykiss	0.5			105		
D. magna	2.0	99				
D. magna	2.0	99				
D. magna	1.0			100		
D. magna	1.0			101		
S. capricornutum	1.0	100			99	
S. capricornutum	1.0	98			104	

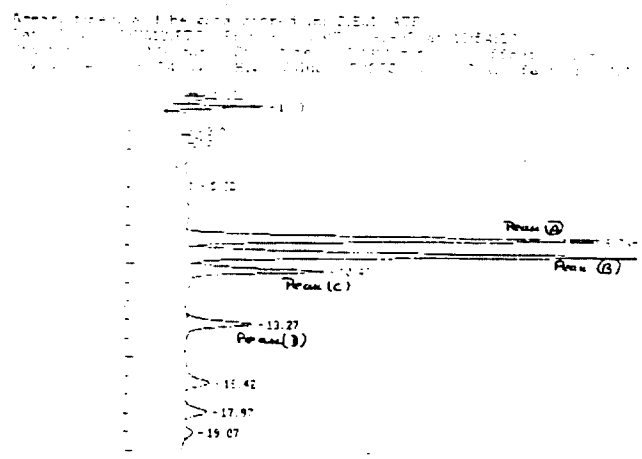
Table D5 - Chromatographic profiles of EPIKOTE 862 standards and of extracts from test media obtained during the D. magna test

Description of sample	Relative proportions of the four major HPLC peaks, assuming equal UV response (%)			
	Peak A(c)	Peak B(c)	Peak C(c)	Peak D(c)
2.5 µg/ml std. (a)	40.0	41.4	12.3	6.3
10 µg/ml std.(a)	40.4	40.9	11.9	6.8
Fresh 1 g/litre extract	37.5	44.3	15.4	2.8
Fresh 500 mg/litre extract	36.9	44.7	15.4	3.0
Fresh 125 mg/litre extract	36.9	44.3	15.6	3.2
Fresh 31.3 mg/litre extract	37.1	44.1	15.0	3.8
Old 1 g/litre extract	36.6	44.0	16.3	3.2
Old 500 mg/litre extract	36.1	45.0	16.8	2.1
Old 125 mg/litre extract	35.3	45.0	16.6	3.1
Old 31.3 mg/litre extract	33.9	45.8	17.3	3.0
2.5 µg/ml std. (b)	40.4	41.7	12.4	5.6
15 µg/ml std.(b)	40.2	40.7	12.2	6.9

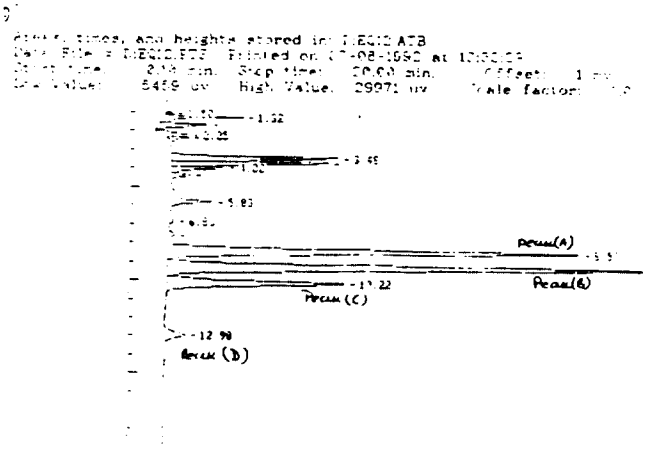
(a) = run with the fresh extracts
(b) = run with the old extracts
(c) = see figure D1 for the positions of these peaks in the chromatogram.

Figure D1 - Typical chromatograms obtained during the analysis of test media for EPIKOTE 862

(a) Standard solution containing 10 μg/ml of EPIKOTE 862



(b) Extract from freshly-prepared 125 mg/litre solution



(c) Extract from 72 hour old 125 mg/litre solution

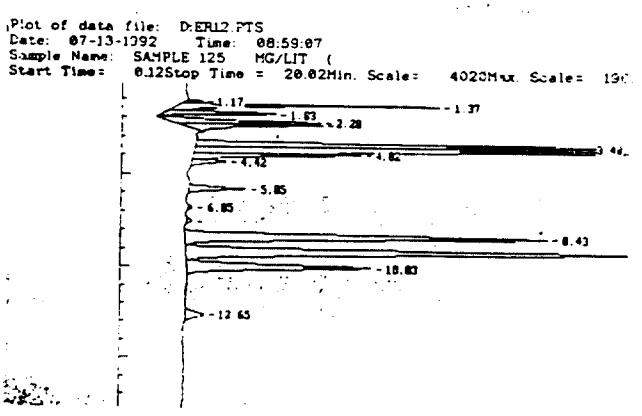
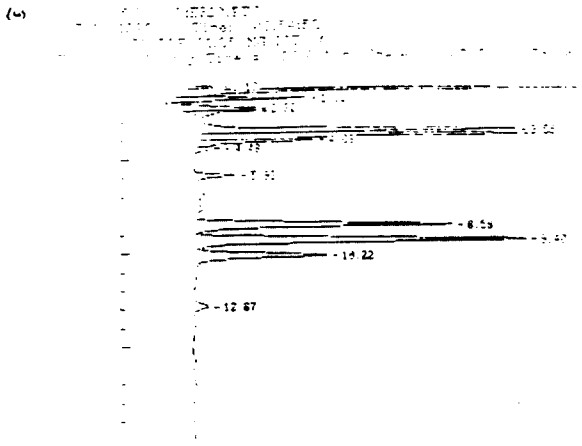
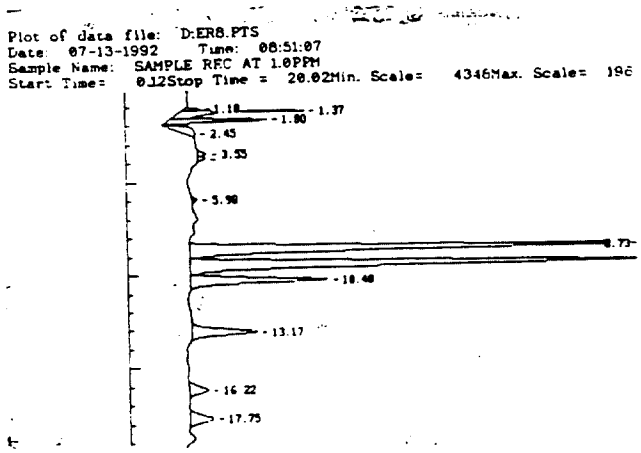


Figure D2 - Typical chromatograms obtained during the analysis of test media for EPIKOTE 862

(a) Extract from 72 hours old 31.25 mg/litre solution



(b) Extract from analysis recovery containing 1.0 mg/litre of EPIKOTE 862.



ATTACHMENT D1

METHOD FOR DETERMINING EPIKOTE 862 IN WATER

1. Summary

This method is intended for the determination of Epikote 862 in water at concentrations down to less than 0.01 ppm m/m. The lower limit of determination has not been established.

A Whatman ODS-2 octadecyl solid phase extraction cartridge, (200 mg/3 ml), is pre-washed with acetonitrile, 5 ml, followed by distilled water, 20 ml. A 100 ml portion of the aqueous sample is added to the reservoir and passed through the cartridge at about 2 ml/min, followed by distilled water, 5 ml. The eluate is discarded. The test substance is eluted from the cartridge with acetonitrile, 5 ml, which is collected, adjusted to a known volume, and analysed by HPLC.

2. Method

2.1 Sample Extraction

Because Epikote 862 will react slowly with water, the sample must be extracted immediately and not stored. For each sample, take a 3 ml ODS 2 octadecyl Whatman solid phase extraction cartridge. Elute it with 5 ml of HPLC grade acetonitrile followed by 20 ml of distilled water. Discard the eluates.

Attach a sample reservoir onto the cartridge, then add a portion of the well mixed sample of water. For samples containing nominal concentrations of 0.2 ppm or less of Epikote 862, a 100 ml portion is required. For concentrations greater than 0.2 ppm, smaller portions are used. Pass the water through the cartridge at about 2 ml/min, then pass through 5 ml of distilled water. Discard the eluate. Add HPLC grade acetonitrile, 5 ml, to the reservoir, and pass it through the cartridge at about 2 ml/min. Collect the eluate in a 5 ml measuring cylinder and adjust the volume as necessary to give extracts within the range of the standard solutions (see 2.3).

Along with each batch of samples, carry out the procedure with water to which an accurate amount of the test substance has been added, for recovery determination. Solutions containing approximately 0.2 mg/litre of the test substance, made by adding a standard solution of the test substance in acetonitrile to water, are convenient for the latter purpose.

2.2 HPLC Conditions

The following HPLC conditions are suitable for analysis of the sample extracts.

Column	:	250 mm by 4.4 mm ID Lichrocart Cartridge packed with Supersphere 100 RP 18.
Mobile Phase	:	60% Acetonitrile in water (v/v)
Flow	:	1.0 ml/min.
Injection Vol.	:	50 µl
Detector	:	Ultraviolet absorption at 230 nm.

Using the above conditions the chromatogram has nine peaks, out of which the first 4 major peaks are monitored to determine concentrations of Epikote 862. The retention time depends on the condition and age of the column.

2.3 Measurement procedure

Calibrate the HPLC system by injecting standard solutions of the test substance in acetonitrile. Primary standard solutions are made by dissolving the appropriate substance in acetonitrile to produce solutions containing 1.0 and 0.5 mg/ml. They are stable in the dark at 4°C, for one month. The range of standards needed will depend on the sensitivity of the HPLC equipment, but solutions containing 2.5 to 15.0 µg/ml of Epikote 862 are normally suitable. These are stable for one week when stored at 4°C in a refrigerator. When a linear response has been established using the first four peaks, inject samples and standard solutions in an interspersed manner. Measure the concentration of Epikote 862 in the extracts from samples by comparing the sizes of the four major peaks in standards with those from samples. Measurements can be on a peak to peak basis, in which case there will be a nominal concentration for Epikote 862 based on each of the four peaks, or on the total peak area for the sum of the four peaks, in which case only one concentration value is obtained. The latter method was used in this study.

Calculate the concentrations of the test substance in the original water sample by applying a correction to take account of the concentration factor produced by the method.

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SBGR.92.237

FURTHER DETAILS FOR DATA BASE ENTRY

INDEX TERMS:

10. CHEMICALS
15. TOXICOLOGY
16. RESEARCH & DEVELOPMENT

KEYWORDS:

EPIKOTE 862, Acute, Aquatic, Toxicity, Fish, Oncorhynchus mykiss, Invertebrate, Daphnia magna, Algae, Selenastrum capricornutum

SBGR.92.237 CONFIDENTIAL

EPIKOTE 862: Acute toxicity to Oncorhynchus mykiss, Daphnia magna and Selenastrum capricornutum

DISTRIBUTION

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SICC (CMSE/2)	2
SICC (CBDR)	1
SICC (CMSE/321)	1
SICM (CMFS/234)	5
ORC (RSOK/7)(via Calgary)	1
Shell Dev. Co. (SDWR)	9
SIPM (HSE/51)	2
KSLA (ICS/1)	2

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Date sent to triage: OCT 2 0 1994

NON-CAP CAP

Submission number: 12724C

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CECATS\TRIAGE TRACKING DBASE ENTRY FORM

CECATS DATA:
Submission # 8EHQ-1093-12724 SEQ. 6

TYPE: [redacted]-SUPP FLWP

SUBMITTER NAME: Shell Oil Company

INFORMATION REQUESTED: FLWP DATE:
0501 NO INFO REQUESTED
0502 INFO REQUESTED (TECH)
0503 INFO REQUESTED (VOL ACTIONS)
0504 INFO REQUESTED (REPORTING RATIONALE)

DISPOSITION:
(0639) REFER TO CHEMICAL SCREENING
0678 CAP NOTICE

VOLUNTARY ACTIONS:
(0401) NO ACTION REPORTED
0402 STUDIES PLANNED/UNDERWAY
0403 NOTIFICATION OF WORKERS/OTHERS
0404 LABEL/MSDS CHANGES
0405 PROCESS/HANDLING CHANGES
0406 APP/USE DISCONTINUED
0407 PRODUCTION DISCONTINUED
0408 CONFIDENTIAL

SUB. DATE: 10/08/93 OTS DATE: 10/13/93 CSRAD DATE: 08/01/94

CHEMICAL NAME: Epikote Resin 862

CAS# 28064-14-4

INFORMATION TYPE:		P F C	INFORMATION TYPE:		P F C	INFORMATION TYPE:		P F C
0201	ONCO (HUMAN)	01 02 04	0216	EPI/CLIN	01 02 04	0241	IMMUNO (ANIMAL)	01 02 04
0202	ONCO (ANIMAL)	01 02 04	0217	HUMAN EXPOS (PROD CONTAM)	01 02 04	0242	IMMUNO (HUMAN)	01 02 04
0203	CELL TRANS (IN VITRO)	01 02 04	0218	HUMAN EXPOS (ACCIDENTAL)	01 02 04	(0243)	CHEM/PHYS PROP	01 02 04
0204	MUTA (IN VITRO)	01 02 04	0219	HUMAN EXPOS (MONITORING)	01 02 04	0244	CLASTO (IN VITRO)	01 02 04
0205	MUTA (IN VIVO)	01 02 04	(0220)	ECO/AQUA TOX	01 (02) 04	0245	CLASTO (ANIMAL)	01 02 04
0206	REPRO/TERATO (HUMAN)	01 02 04	0221	ENV. OCCC/REL/FATE	01 02 04	0246	CLASTO (HUMAN)	01 02 04
0207	REPRO/TERATO (ANIMAL)	01 02 04	0222	EMER INCI OF ENV CONTAM	01 02 04	0247	DNA DAM/REPAIR	01 02 04
0208	NEURO (HUMAN)	01 02 04	0223	RESPONSE REQEST DELAY	01 02 04	(0248)	PROD/USE/PROC	01 02 04
0209	NEURO (ANIMAL)	01 02 04	(0224)	PROD/COMP/CHEM ID	01 02 04	0251	MSDS	01 02 04
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0213	SUB ACUTE TOX (ANIMAL)	01 02 04	0228	ALLERG (ANIMAL)	01 02 04			
0214	SUB CHRONIC TOX (ANIMAL)	01 02 04	0239	METAB/PHARMACO (ANIMAL)	01 02 04			
0215	CHRONIC TOX (ANIMAL)	01 02 04	0240	METAB/PHARMACO (HUMAN)	01 02 04			

TRIAGE DATA:	NON-CBI INVENTORY	ONGOING REVIEW	SPECIES	TOXICOLOGICAL CONCERN:	USE:	PRODUCTION:
	(YES)	YES (DROP/REFER)	Fish	LOW	Resin & monomer	
CAS SR	NO	NO (CONTINUE)	Daphnia magna	MED		
	DETERMINE	REFER:		HIGH		

COMMENTS: Non-CAP, SS